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CONTENTS OF VOLUME III

THE ACTION OF MUSCARIN AND PILOCARPIN ON THE HEARTS OF CERTAIN VERTEBRATES, WITH OBSERVATIONS ON SEASONAL CHANGES

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Whether muscarin and pilocarpin, when applied to the heart, act directly in virtue of their depressing influence on the inherent motor activity, or indirectly as excitants of the cardio-inhibitory mechanism, is still an open question.

Schmiedeberg and Koppe[1] attributed the action of muscarin to its influence as a stimulant of inhibitory ganglia. Kobert[2] subsequently emphasised the same idea. Later on Schmiedeberg's general results were confirmed by Prevost and Monnier[3]. Since that time many investigators have published results favouring the theory of vagal stimulation, and many authorities of the present day accept the view that these drugs act by stimulating the vagus endings.

On the other side, the direct action of a comparatively weak solution of muscarin or pilocarpin as a paralyser of the cardiac muscle substance, has been emphasised by various authorities.

One of the strongest advocates of the theory of direct depression of the cardiac motor mechanism is Gaskell[4]. After the application of muscarin he found a general depression of rhythm, strength, tone and conductivity. He holds that the drugs act on the muscular tissue atonically.

1. Schmiedeberg u. Koppe, *Das Muscarin*, Leipzig, F. C. W. Vogel, 1869, S. 28; *Studien uber Herzgifte*, Würzburg, 1871.

2. *Archiv. f. exper. Pathol. u. Pharmakol.*, Bd. 20, S. 92.

3. *Gaz. méd. de Paris*, 1874, p. 243.

4. *Philosophical Transactions*, 1882. *Journal of Physiology*, Vols. III, IV, and VIII. *Schäfer's Text-book of Physiology*, (1900) p. 223.

Description of Samples of Muscarin and Pilocarpin used in this Investigation

In all investigations bearing on the action of muscarin with regard to cardiac inhibition, it is obviously of primary importance that the drug should be pure. Schmiedeberg has pointed out that the samples obtained from dealers are never sufficiently purified, and are liable to contain, amongst other impurities, traces of an atropin-like base. The solution used in these experiments has been kindly supplied by Professor Schmiedeberg himself, and a similar solution has been used by Gaskell in his experiments on muscarin action. The latter observer found that the strength was such that one or two drops placed on the sinus of the frog's heart were sufficient to cause immediate standstill; placed on the sinus of the tortoise, the beat ceased immediately[1].

With this solution of muscarin the majority of my experiments were made; in suitable cases one drop or even less applied to the sinus gave the characteristic effect; if one or two drops were not sufficient, an increase in the quantity never gave an immediate effect, the result of the exhibition of a large quantity being essentially different in nature from that of a small amount.

For some experiments pilocarpin was used, and in order to insure the elimination of any possible errors arising from impurities or defects in the salts, five different specimens were used—three nitrates and two chlorides—two of these being specially certified to contain no impurities. The solutions were made up in Ringer's circulating fluid, so that 1 drop as obtained from a special pipette contained exactly 2 mgrs. of the salt; by dilution it was thus easy to apply to the heart any fraction of a mgr. of the pilocarpin. The activity and purity of the samples used, I have often demonstrated on the normal heart of the frog and eel, when a small quantity applied to the sinus produced the usual results.

1. *Journal of Physiology*, Vol. VIII, p. 407.

Heart of Frog

On Seasonal Changes in the Frog-Heart, and their Influence on Inhibition.—When making observations on the heart of the British frog in the months of January, February and March, I was surprised to find that very considerable changes were in evidence with regard to the manner in which the heart responded to vagus stimulation. In some cases the inhibitory function of the vagus seemed to be quite inactive, while in others it was manifested only to an exceedingly slight degree. In January, certain hearts responded to faradisation applied to the 'posterior white crescent,' but in many the vagus influence seemed to be very slight indeed, the heart quickly escaping from the effects of stimulation and regaining its former rhythm; on the application of a stronger faradic stimulus there was occasionally a slight inhibitory effect, but it was always of very short duration, and soon no strength of stimulus had any effect. Stimulation of the vagus trunk or medulla did not, as a rule, alter in any way the rate of cardiac action, though in a few cases there was a rather ambiguous result produced probably dependent on the intermixture of accelerator and inhibitory fibres in the frog's vagus (*Rana temporaria*).

In February and March the same features were in evidence, only the elimination of vagus influence became gradually more pronounced, till towards the end of March it often happened that no inhibitory effect could be demonstrated. In April, also, there was little response to vagal stimulation, but in May the heart was found to be gradually assuming its normal condition, for faradisation of the posterior white crescent often resulted in a marked slowing or temporary stoppage of the heart; even in May, however, normal conditions did not by any means prevail.

In the autumn months the result of vagus stimulation on British frogs was quite different, the majority giving unmistakable evidence of an active inhibitory mechanism; some German frogs examined in October, however, gave no result, and it is interesting to note that these were distended with ova (*Rana esculenta*).

Many observers have noted certain changes in the ordinary

frog-heart during the winter and spring months, but there does not seem to be any definite account of the very marked phenomenon in regard to this diminution of the inhibitory power, and the exact cause of it is difficult to establish.

A striking fact is the observation that in a general way this change coincides more or less with the season of sexual activity in the frog, while such factors as temperature and the diminished activity of the animal in the cold season may also have some influence. Ringer[1] noticed that certain antagonisms were slight during the breeding season. Pandelejeff[2] also found that the antagonism of atropin for quinine on the frog-heart was affected by the time of year. In summer frogs, quinine arrested the heart in diastole and atropin caused the heart at once to resume its pulsations. In winter frogs, quinine arrested the heart much more slowly, while atropin, instead of obviating actually increased the arrest. Low temperature would not entirely account for the changes observed in regard to inhibition, for while the mean temperature in January was fully 6° F. lower than that in the beginning of April, yet these changes were much more pronounced in April. Sexual activity in the frog at any rate seems to be the dominant factor in the production of this change. Jordan found the action of muscarin difficult to demonstrate in winter frogs.[3]

The action of Muscarin and Pilocarpin on the Frog-Heart affected by Seasonal Changes.—The diminution, or in some cases, absence of any apparent inhibitory action in response to stimulation of the cardio-inhibitory apparatus in frogs affected by the seasonal changes discussed above suggested the idea that advantage might be taken of the condition in order to test the mode of action of muscarin and pilocarpin. Here was a case where in certain frogs there was a comparative elimination of the function of inhibition, while in others it was markedly less than normal, probably an expression of some profound general change.

1. *Journal of Physiology*, Vol. III, p. 115.
2. *Lancet*, July 31st, 1880, p. 176.
3. *Arch. f. exp. Pathol. u. Pharmakol.*, VIII, 1878.

If muscarin stopped or slowed the heart in these frogs, then this drug could not be acting through a medium which had been proved inactive by other means. In other words, if muscarin acted as an excitant of the cardio-inhibitory nerves, its effect on the cardiac rhythm should bear some comparatively constant ratio to the effect of electrical stimulation of the inhibitory apparatus. If, on the other hand, muscarin acted as a direct depressant of the inherent motor mechanism of the cardiac muscle, then the fact of the inhibitory apparatus being inert should not influence its action when applied to the heart substance. During a long series of experiments carried out at different seasons during the last three years, it was found invariably that when electrical stimulation of the inhibitory mechanism through the *posterior white crescent* failed to affect the rhythm of the heart, then no effect was produced by the application of weak solutions of muscarin or pilocarpin. When electrical stimulation gave a positive result (*i.e.* slowed the heart) muscarin also gave a like result in many cases ; as a general rule, faradisation quickly resulted in tiring out the already attenuated inhibitory apparatus so that subsequent increased stimulation had no effect, and in such a case muscarin likewise proved ineffective ; if the faradisation was immediately stopped after an effect was observed, the subsequent application of muscarin generally resulted in slowing the heart for a time ; an escape from the inhibition, however, generally occurred in a comparatively short time ; this setting free of the heart could be brought about by faradisation and by muscarin or pilocarpin.

Even in late summer and autumn when normal conditions apparently prevail, many variations were met with in the course of this investigation, but no instance was observed in which a heart unaffected by faradisation of the posterior white crescent gave any result in the direction of slowing or stoppage after the exhibition of muscarin.

Fig. I[1] represents a tracing from a 'normal' frog heart, showing result of a local application of a few drops 2 per cent. pilocarpin

1. All the tracings read from left to right.

solution; heart is seen to be considerably slowed. The result of atropin is also well seen. Drum was stopped for a few seconds on each application to give drugs time to act.

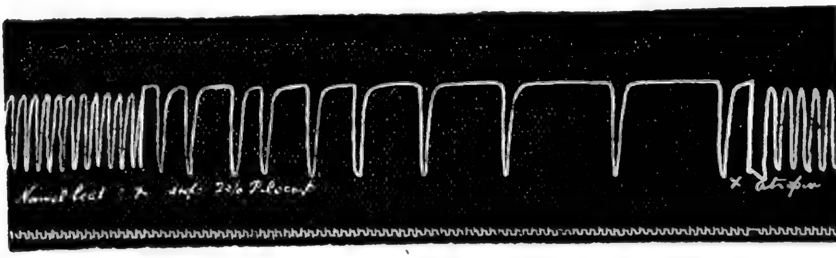


Fig. I. Effect of pilocarpin on frog's heart. (Autumn).

Fig. II is from an 'abnormal' heart in which faradisation failed to cause inhibition, showing result of pilocarpin application. Here such a strong solution as 4 per cent. pilocarpin failed to influence the beat even after ten minutes.

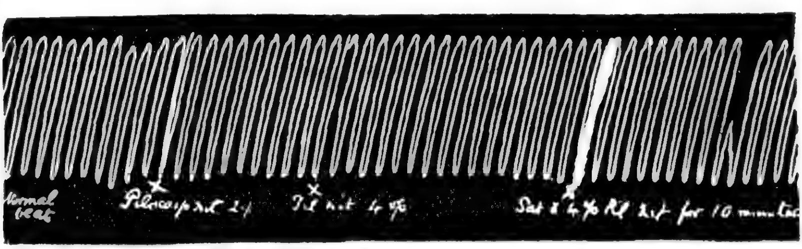


Fig. II. Effect of pilocarpin on frog's heart. (Spring).

With large doses of muscarin or pilocarpin, direct depression of the properties of cardiac muscle became evident; the time and amount of drug required for this varies greatly in different frogs according to the vitality of the heart and animal in general. In nearly all my experiments the depression of these special functions of cardiac muscle followed a well marked rule. The first effect of a large dose seemed to be on the muscular *tone*. Very soon after the application of a strong solution of pilocarpin the whole heart became somewhat flabby, both the auricle and ventricle exhibiting well marked distension during systole. This did not seem to influence

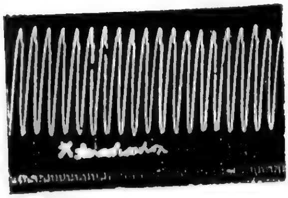
immediately the rate or force. Some time afterwards the rate became less, due probably to a diminution of *excitability*. A weakness of the cardiac *contractile force* was next manifested, followed often by a condition in which a considerable interval elapsed before the *conduction* of the auricular beat to the ventricle was affected. The order, therefore, in which pilocarpin seems to depress the properties of cardiac muscle is—

Diminution in (1) Tone
(2) Rate
(3) Contraction force
(4) Conduction.

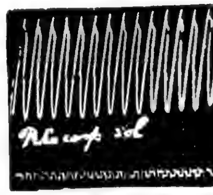
A heart slowed by direct depression by a large dose of pilocarpin acting for a comparatively long time, differs from a heart quickly arrested or slowed by a small dose in the fact that the rate of the former heart is not increased by atropin, whereas digitalin immediately caused a marked acceleration. On a heart quickly slowed by a small dose, digitalin in weak solution has little or no effect when the inhibition is strong, but atropin gives a most marked and immediate result.

Several of the above points are seen in Fig. III. In A faradisation was applied at the point marked, but without effect. B was taken half a minute after the application of a few drops 2 per cent. pilocarpin; no change is apparent. The heart was then covered with 5 per cent. pilocarpin nitrate solution and left for 25 minutes (*see* C, D, E); it was then again covered with pilocarpin solution every 5 minutes until 65 minutes had passed; gradual slowing due to direct muscular depression is seen, but this is not marked till after one hour or so. After 65 minutes, atropin was applied (E): heart still slow. Four minutes after first dose of atropin more was applied, the result being to cause more marked slowing. Digitalin in weak solution had a marked effect, the rate being instantly quickened (F).

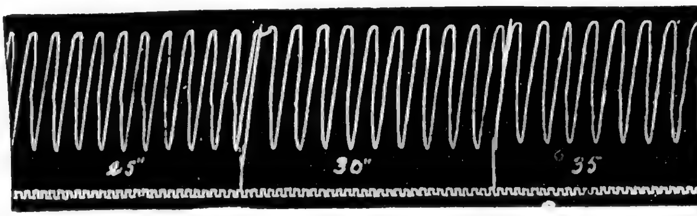
In a strong frog the heart may often be soaked with pilocarpin nitrate, from 5 to 10 per cent. solution, and yet be able to beat quite strongly after an hour or more, though in direct contact with the



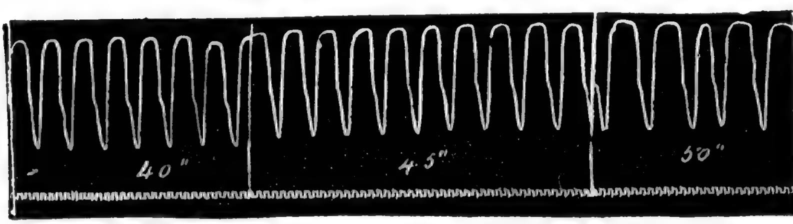
Faradisation
A



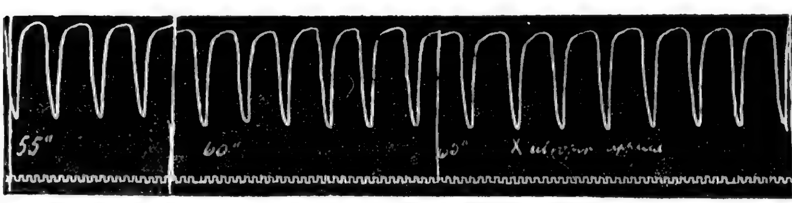
Pilocarp. sol.
B



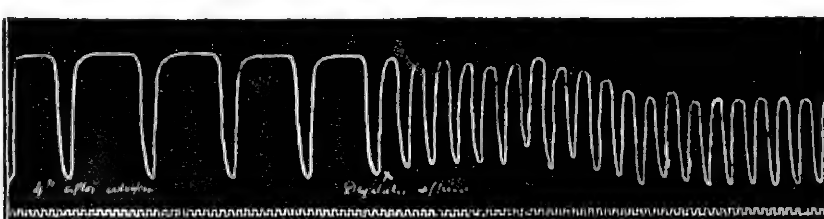
25′ 30′ 35′
C



40′ 45′ 50′
D



55′ 60′ 65′ Atropin applied
E



4′ after atropin Digitalin applied
F

Fig. III.

Direct weakening action of pilocarpin after long period, with effect of atropin and of digitalin.

drug the whole time; in general a diminution in rate is observed in much less than an hour.

Weaker frogs vary considerably in regard to the time and amount of drug required to cause depression, but they never give an indication of depression unless the dose is much greater than is usually necessary to produce a result in a normal frog-heart—also the time necessary for the occurrence of appreciable direct depression is much greater than is generally required for the causation of slowing or stoppage in a normal heart.

The following experiments serve as an indication of the condition obtaining in certain frogs during the months when sexual activity is most manifest; it will be noticed that in the cases quoted, inhibition was often found to be practically absent; such cases serve best to bring out a certain phase of the parallel action of muscarin and faradic stimulation. In many experiments there was, of course, a certain amount of inhibitory power present, but as a general rule, as the result of a great number of experiments repeated during several years, it was found to be markedly diminished during the spring season. In all experiments mentioned in this paper, faradisation was applied to the 'posterior white crescent.'

Experiments

Experiment I.—January.

Remarks on heart	Drug	Dose	Faradisation	Rate before (per minute)	Rate after (per minute)	Condition of heart
Exposed in in situ; fairly vigorous	Muscarin	—	Negative	22	—	—
		1 drop	...	...	22	Strong beat
		1 „	...	...	22	„ „
		2 drops	...	...	20	—
		4 „	...	...	21	Heart quite strong after 8 drops After 10 minutes rate 18 per minute and fairly strong; gradually became weaker
	Atropin	2 drops 1 % sol.	...	...	14	Weaker still
	Digitalin	Few drops of weak solution	...	...	23	Slightly stronger beat

Experiment II.—February.

Remarks on heart	Drug	Dose	Faradisation	Rate before (per minute)	Rate after (per minute)	Condition of heart
Exposed in situ; strong beat	Pilocarpin nitrate (3 %)	—	Stopped heart for 10 seconds; afterwards failed to affect it	30	—	—
		1 drop	...	...	30	—
		1 ,,	...	...	31	—
		1 ,,	...	...	27	Quite strong
		1 ,,	...	...	30	—
		1 ,,	...	...	29	—
		2 drops	...	...	25	—
		3 ,,	...	...	26	—
		4 ,,	...	...	26	—
		5 ,,	...	...	25	Quite strong After ½ hour beating strongly 18 per minute Digitalin increased rate to 25 per minute

Experiment III.—March.

Excised; feeble heart	Muscarin	—	Negative	18	—	—
		½ drop	...	...	18	Weaker
		1 ,,	...	...	18	Still getting weaker
		1 ,,	...	..	16	—
		1 ,,	...	...	14	Very weak; soon only faint contractions noticed passing over heart; then stopped Digitalin no effect

4′ a1

Direct weakening ac

Experiment IV.—April.

Remarks on heart	Drug	Dose	Faradisation	Rate before (per minute)	Rate after (per minute)	Condition of heart
Excised; fairly vigorous heart	Muscarin	—	Negative	16	—	—
		$\frac{1}{5}$ drop	...	...	16	—
		$\frac{2}{5}$ "	...	...	14	—
		$\frac{3}{5}$ "	...	...	14	—
		$\frac{4}{5}$ "	...	...	16	—
		1 "	...	...	16	—
		1 "	...	...	13	—
		1 "	...	...	12	Beat rather weak
		1 "	...	...	15	—
	Atropin (1 % sol.)	3 drops	...	...	12	Beat still weaker; heart placed in muscarin solution, continued to beat though weakly for several minutes; washed and treated with Atropin —no change
						Helped slightly by digitalin

Experiment V.—May.

Remarks on heart	Drug	Dose	Faradisation	Rate before (per minute)	Rate after (per minute)	Condition of heart
Exposed in situ; strongly beating	Muscarin	—	Slowed to average of 10 per minute	30	—	—
		$\frac{1}{2}$ drop	...	...	24	Strong
		1 "	...	...	13	—
		1 "	...	...	21	Strong
		1 "	...	...	25	—
		1 "	...	...	20	Slightly weaker
		2 drops	...	...	20	—
		1 drop	...	...	18	Somewhat weaker but beating after other 6 drops
						Atropin—no effect
						Digitalin—beat quicker and stronger

On the Antagonism of Muscarin and Pilocarpin

Many years ago Ringer[1] concluded that pilocarpin antagonizes the action of extract of muscarin on the frog-heart, a curious result in regard to two drugs exhibiting such closely allied pharmacological action as pilocarpin and muscarin. Judging from the general action of these two drugs it would be expected that a heart slowed by a certain amount of muscarin would be further slowed by an additional dose of pilocarpin. Instead of this, however, Ringer has found that in certain cases, a heart slowed by muscarin is accelerated by the application of pilocarpin, and he therefore concludes that pilocarpin acts as an antagonist to muscarin.

This curious phenomenon I have observed on several occasions, and the idea suggested itself that if muscarin acts on the cardio-inhibitory nerves, and not on the muscle directly, an explanation of this action might be afforded by the fact that the primary dose of muscarin resulted in a stimulation of the nervous mechanism, and thus brought about a slowing of the heart, whilst the subsequent acceleration of cardiac action by pilocarpin might be due to the increased dose paralysing the inhibitory mechanism to a greater or less extent and thus increasing the rhythm. In a heart where the inhibitory function was but little marked, this would probably happen. If this was the case the same result should be brought about by the application of more muscarin after the preliminary slowing by that drug. In the course of my experiments it was very obvious at certain seasons that a heart slowed by muscarin in small dose was, on the application of a little more of the drug with a view to increasing its inhibitory action, accelerated instead of slowed, and the same result was even more frequently obtained with pilocarpin. This was not so much in evidence with muscarin, at any rate in the normal frog-heart, and here it would seem that a dose of the drug sufficient to paralyse the inhibitory apparatus is so great as to directly depress the muscle. In this case no result could be expected. Again, at the season of increased sexual activity, when

1. *Journal of Physiology*, Vol. II, p. 135.

the inhibitory mechanism was practically functionless, this effect was not obtained ; any depression of rhythm in this case, however, must have been due to the effect of a large dose in depressing the cardiac muscle, and so no subsequent acceleration could be expected ; the phenomenon was best seen in a heart where inhibition was feeble but still active. The above observations do not account for many of the results obtained by Ringer, and are not inconsistent with the idea of the existence of a real antagonism between the two drugs ; on the other hand, it is obvious that the phenomenon described above would probably be accepted as a true antagonism, whereas it really is but an apparent one.

Here the escape of the heart from the effects of muscarin while the drug is still in contact with the cardiac tissue admits of practically the same explanation, and the fact that within certain limits this escape can often be brought about by the application of an increased amount of the original drug, seems to be inconsistent with the idea of direct muscular depression ; if, however, too much muscarin be added, depression of the muscle may of course be evident, and so prevent increase in the rhythm, while if the increased dose is too small to paralyse the mechanism, the inhibitory effect is more marked, as generally occurs in the normal frog-heart.

In view of these observations, it would seem that in certain hearts at any rate the apparent antagonism of muscarin and pilocarpin is due to the condition of the cardio-inhibitory apparatus.

Again, if muscarin produces its effects by its direct action on the cardiac muscle, it is difficult to understand how it should sometimes have its apparent effects considerably lessened by an additional dose.

On a Tendency for the Establishment of Immunisation towards Muscarin and Pilocarpin in the Frog-Heart

A very important factor exhibited in the frog-heart is a tendency for the production of a certain amount of immunisation to muscarin or pilocarpin after the exhibition of these drugs. When the heart recovers from the effect of a small dose of muscarin, as often happens

in certain hearts, it was found that a very much greater dose of the drug was required to produce slowing or stoppage a second time; in many cases, as previously stated, it was not possible to produce a second effect, but in a fairly normal heart this could sometimes be done. I have never seen an instance of any heart escaping from the effect of a second dose being slowed by the application of more of the drug, except in cases where, after a comparatively long time, direct muscle weakening set in. It is interesting to note that many of the above results agree with observations made by Marshall on the mammalian heart[1]. In view of what we know of the behaviour of muscle towards depressant drugs, such immunisation would be difficult to explain if muscarin (or pilocarpin) is in small doses a direct muscle depressant, but is perfectly intelligible if the drug is a stimulant to nerve endings[2]—repeated doses leading to exhaustion of their function. That the action of pilocarpin is on nerve-endings, however, has been comparatively recently disputed by Matthews[3], who still advocates direct action on the animal cell, at least in the case of gland-cells.

Heart of Eel

The heart of the eel, as shown by MacWilliam[4] many years ago, possesses a very marked susceptibility to vagus inhibition, and differs from the frog-heart both as regards the state of the cardiac tissue while under the influence of vagal stimulation and in the peculiar manner in which spontaneous cardiac rhythm becomes re-established.

On Seasonal Variations in the Eel-Heart.—Here, as in the heart of the frog, great variations in susceptibility are met with at different seasons. In some experiments done on eels

1. *Journal of Physiology*, Vol. XXXI, p. 127. The above observations were presented as part of a thesis to the Senatus of the University of Aberdeen before I knew of Marshall's paper.

2. In this paper the term 'nerve endings' is used to indicate the peripheral terminations of the cardiac inhibitory apparatus. The present investigation shows that the drugs mentioned act indirectly by stimulation of a peripheral inhibitory mechanism and not directly by simple depression of the contractile mechanism; whether the exact portion of this inhibitory apparatus is of nervous or muscular origin is not within the scope of the investigation. It is very possibly intra-cellular.

3. *American Journ. of Physiol*, Vol. VI, p. 207, 1901.

4. *Journal of Physiology*, Vol. VI, p. 218.

obtained about the beginning of March, it was found that in every case faradisation of the sinus or vagus nerve had little or no effect on the cardiac rhythm or contractile force; in twelve eels experimented upon not one was definitely influenced by faradisation; here again muscarin and pilocarpin proved equally ineffective, and comparatively large doses not only failed to influence the rhythm, but a heart covered with such strong solutions as 4 per cent. pilocarpin nitrate or chloride for several minutes, showed no appreciable diminution in the force of the cardiac contraction.

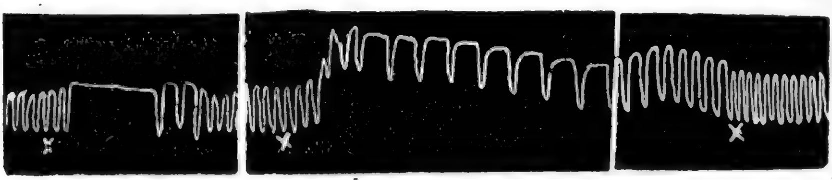
In eels obtained early in April the same phenomenon was in evidence in some of them; in a certain number of these, however, fairly definite results could be obtained by faradisation as well as by the application of muscarin and pilocarpin, but here the heart could not be always brought to a standstill by faradisation, though a considerable slowing was often observed; if the faradisation was applied for even a comparatively short time, the heart generally succeeded in escaping from the inhibitory influence and regained its normal rate; the application of a stronger stimulus sometimes gave a result, but it was always less than that obtained from the primary stimulation, and soon the heart escaped and could not be again influenced by vagal excitation. After this faradisation the heart was quite uninfluenced by muscarin or pilocarpin even in large amounts, and the fact that in these hearts the usual effects of the drugs can be prevented by previous faradisation of the vagus seems to prove that muscarin and pilocarpin are directly dependent on a functionally intact inhibitory mechanism for their action in slowing or stopping the heart.

Again, the effect of muscarin and pilocarpin always ran parallel with that of faradisation; in several cases a heart which had escaped from fairly weak faradisation was slowed by pilocarpin, but the effect very soon passed over, the heart regaining its former rhythm; when this happened, pilocarpin, even in large doses, was quite ineffective.

Fig. IV shows a tracing from such a heart. In A is seen a stoppage from weak faradisation, which was applied at the point marked on the left. After a short initial stoppage, beat escaped though current still applied. B is a continuation of the same tracing showing

result of application of pilocarpin. (The difference in level is due to a reflex movement of the eel).

In C the pilocarpin is almost recovered from ; atropin has been applied at part marked.



A B C

FIG. IV. Effect of faradisation and pilocarpin on a heart in which inhibitory action was present to a slight degree.

In these hearts it was obvious that vagal inhibitory power was present only to a slight extent, and this inhibition seemed to be still less in evidence if the heart was injured in the process of exposure ; an excised heart at this season seldom gave any definite indication of inhibition either as the result of faradisation or drugs, and it would seem that even in hearts in which inhibition is very pronounced, mechanical injury tends to diminish the phenomenon.

More eels were obtained at different seasons later on, and it was found that normal conditions became gradually established. In May, however, many hearts were obtained that seemed to possess no very marked inhibitory mechanism ; here the same relationship as above stated obtained as regards faradisation and the application of muscarin and pilocarpin. In July, some eels were examined and found to be almost normal, though inhibition was not so marked in all as it appeared to be later.

In September and October, inhibition was found to be much more active, and both faradisation and muscarin or pilocarpin instantly stopped the heart for a long period ; the application of atropin immediately restored cardiac action.

Fig. V shows a tracing taken from a normal eel-heart showing effect of pilocarpin : drug was applied at part marked on left and drum

stopped for a few seconds; single ventricular beat in middle shows result of mechanical stimulation. On the right is seen the effect of atropin; here drum was also stopped for a few seconds.

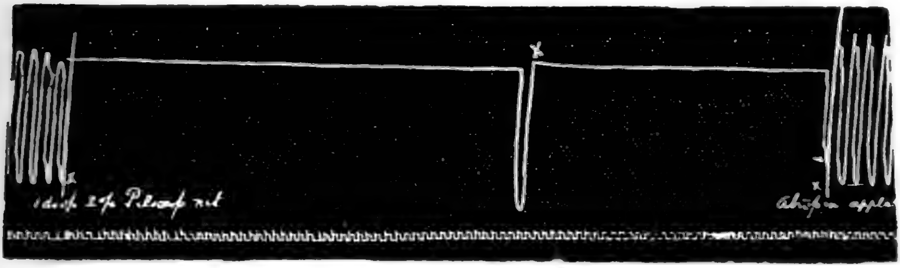


FIG. V. Effect of pilocarpin on normal heart.

At all seasons of the year a heart may occasionally be found in which faradisation of the vagus gives no result, and in such a case muscarin and pilocarpin are likewise incapable of slowing or arresting the rhythm. This close relationship between the effects of faradisation and the application of muscarin or pilocarpin can only be rationally explained by assuming that these drugs act by stimulation of the cardiac inhibitory nerves.

As in the case of the frog-heart this difference in regard to inhibition at different seasons is difficult to account for, but it is likely that the same general causes may be present as factors in both cases.

On the Condition of the Eel-Heart when brought to a standstill by Faradisation of the Vagus and by Muscarin or Pilocarpin.—In the normal eel-heart arrested by faradisation of the vagus, the ventricle generally remains quite responsive to stimulation, while the auricle remains inexcitable: the same thing commonly obtains in the eel-heart arrested by moderate doses of muscarin or pilocarpin; the ventricle responds readily to stimulation while the auricle remains quiescent. In many other points also, the eel-heart behaves in a peculiar manner while under the influence of vagus inhibition, and the same conditions seem to obtain under the

influence of muscarin and pilocarpin; these points are at present being investigated.

The following are notes of a few of the experiments done at different seasons of the year:—

Eel I.—March—

12.0 Heart exposed in situ: rate 28 per minute.
12.5 Faradisation applied to sinus: no result even when strong.
12.8 1 drop muscarin applied to sinus: no result; 21 per minute.
12.12 Another drop applied: rate 20 per minute.
12.20 4 drops applied: heart beating fairly strong; 18 per minute.
12.25 4 drops applied: slightly weaker; 19 per minute.
12.30 Atropin applied, but no change in rate or force; 19 per minute.
4.0 Heart still beating: very weak; 17 per minute.

Eel II.—March—

11.10 Heart exposed in situ: rate 25 per minute.
11.15 Faradisation slowed heart slightly, but only for about 1 minute.
11.20 Pilocarpin nitrate (20 %)—1 drop applied: no result; 25 per minute.
11.24 ,, ,, 2 drops applied: no result; 23 per minute.
11.33 ,, ,, several drops applied: no result; heart quite strong.
11.37 *Heart excised.*
11.40 Pilocarp. applied, many drops: no result; 25 per minute.
11.45 Heart much weaker: 22 per minute.
11.55 Heart getting weaker: 19 per minute; after little time 16 per minute.
11.55 Heart getting weaker: 19 per minute; after little time 16 per minute.
12.5 Atropin applied: no result; 18 per minute and weak.
12.8 Digitalin solution applied: beat stronger; 16 per minute.
12.30 Heart beating, but very feeble: 13 per minute.

Eel I.—April—

10.30 Heart exposed: 19 per minute.
10.33 Faradisation effective; only tried for ¼ minute.
10.38 Pilocarpin applied: beat slowed; rate 14 per minute.
10.41 strong beat; rate 10 per minute.
10.46 More pilocarpin applied: strong beat; rate 12 per minute.
10.55 strong beat; rate 16 per minute.
11.0 beat rather weaker; rate 16 per minute.
11.3 Atropin applied: beat 22 per minute.

Heart continued to beat for over 6 hours.

Eel I.—July—

11.15 Heart exposed: reflex inhibition from gill easily obtained; heart stopped for 65 seconds. Another arrest for 65 seconds occurred when gill again pressed.
Faradisation of sinus: ready arrest of whole heart for 1 minute.

11.27 *Heart excised.*

11.29 Faradisation: arrest of whole heart for 2 minutes 10 seconds; then one beat occurred followed after an interval of 40 seconds by another.

11.37 Faradisation: inhibition for over 3 minutes.

12.5 Faradisation of auricle: arrest of whole heart for 3 minutes (not tried long). Went on all right when current stopped. Heart dull: Ringer's fluid applied.

12.30 Beating very slowly; 4½ per minute.
Ringer's fluid removed to try faradisation: heart did not beat.
" " re-applied: heart again gave auricular beat. Ventricle not beating.

12.35 Faradisation of auricle stopped it for 1 minute (not tried longer): sinus went on beating.

12.42 Faradisation of sinus readily arrested sinus and auricle.

12.46 Pilocarpin readily arrested and atropin restored the beats.

Eel II.—July—

10.16 Heart exposed.

10.19 Reflex inhibition from pressure on gill (1 minute) easily got.

10.25 Faradisation of sinus: arrest of whole heart (for 1 minute).

10.29 Piece of auricle ligatured off with some blood on it.

10.30 *Heart excised.*

10.31 Inhibited from faradisation of sinus.

10.35 Separated piece of auricle began to beat some little time ago—now 6 per second.

10.40 Inhibitory effect of faradisation of excised heart is absent even with fairly strong current.

10.41 Faradisation of separated (autom. beating) auricle does not inhibit. Some exciting effect at beginning.

10.43 Pilocarpine chlor. (1 %) applied to (*a*) excised heart and (*b*) isolated piece of auricle: many drops poured on.

10.47 Pilocarpine has not arrested or apparently depressed (*a*) or (*b*).

10.50 Pilocarpine still no effect.

11.0 Heart still beating at good rate though weaker; separated piece of auricle still beating.

11.5 Fresh blood from another eel (very large) has been applied to separated piece of auricle; now beating rapidly 23 per minute and vigorously: pilocarpine again applied—no arrest.

11.6 Atropin applied to excised heart: no improvement.

11.20 (*a*) and (*b*) still beating; (*a*) weaker and ventricle responds only to every second auricle beat.

12.45 (*a*) and (*b*) still beating though weakly. Pilocarpine no effect.

EEL I.—October—

11.30 Heart exposed: rate 26 per minute.

11.33 Faradisation gave marked result.

11.35 Pilocarpin applied: heart stopped after ½ minute.

11.38 Heart beating slowly: 5 per minute; strong.

11.39 More pilocarpin applied: 3 per minute; strong.

11.42 Atropin applied: 23 per minute (after ½ minute).

11.48 Faradisation negative after atropin.

11.55 Heart at 26 per minute.

After 20 hours—

Heart examined. Sinus 16 per minute (weak); ventricle 6 per minute (also weak).

Pilocarp. nitrate applied (3 drops 2 % sol.). Heart: 3 per minute (ventricle); 9 per minute (sinus).

Atropin sulphate sol. applied: 3 per minute; sinus 9 per minute. Heart very much weaker, and only shows feeble wave of contraction.

EEL II.—October.

11.15 Heart exposed: 28 per minute.

11.16 Faradisation gave marked result—(tried for 1 minute only).

11.20 Applied 1 drop (2 % sol.) pilocarp. nitrate: 12 per minute: strong beat: 4 per minute.

11.23 Applied another drop pilocarp. sol.: 4 per minute.
strong beat: 3 per minute.
fairly strong beat: 3 per minute.

11.41 Applied 2 drops pilocarpin. sol.: 3 per minute.

11.46 Applied 3 drops ,, 3 per minute.

11.48 Heart covered up with blotting paper saturated with pilocarp.

After 2 hours. Solution (2 %). Also many drops poured on heart.

1.48 Beat not very strong: rate 26 per minute.

ON THE DEPRESSANT ACTION OF MUSCARIN AND PILOCARPIN ON THE EEL-HEART

Muscarin.—The sample of muscarin obtained from Professor Schmiedeberg was used, and it was found that the direct depressant effect of the drug on the cardiac muscle, though much greater than pilocarpin, was not very marked, at least for some considerable time,

unless a comparatively large amount had been applied. A normal heart could be arrested by the application of one drop to the sinus region, but hearts in which inhibition was absent were often not affected by many drops, either as regards rhythm or contractile force—in fact it generally required a considerable time (from five to ten minutes or more) for any appreciable diminution to become apparent in the cardiac rate or force. The contrast between the comparatively sudden stoppage or slowing of the normal heart caused by say one drop of muscarin applied to the sinus region, and the very gradual diminution of force and rate noticed only after a considerable time in a heart in which the inhibitory apparatus is functionless, is very marked and suggestive; this gradual weakening is not helped by atropin, but the application of digitalin in weak solution increases the force. On the other hand, digitalin applied to a heart stopped by a drop or two of muscarin in the ordinary way has little effect. These points indicate that the drug must necessarily possess two different modes of action. As the supply of muscarin was limited the majority of eel-heart experiments were performed with pilocarpin.

Pilocarpin.—As the result of very many experiments with pilocarpin, it was found that this drug in doses sufficient to produce profound cardiac inhibition, proved to have only very feeble muscle depressant power; different hearts require different strengths within certain limits, but generally a few drops of a 1 per cent. or 2 per cent. solution of the salt was quite sufficient to produce a marked result in the normal heart. In a heart possessed of but feeble inhibitory power the initial slowing consequent on the application of a few drops of pilocarpin solution was quickly recovered from, and in these cases the application of the drug in quantities sufficient to cover up the heart so that it was beating in a solution of pilocarpin, did not seem to depress it, at least for a very considerable time: the same held true when as much as 30 minims was injected into the circulation. In some hearts covered with 2 per cent. pilocarpin nitrate, the heart was wrapped up with blotting paper saturated with the same solution and left over night; next day it was sometimes found to be beating fairly strongly, though naturally very much weaker than at first;

at other times there was no spontaneous contraction, but there was quite a ready response to mechanical stimulation of the ventricle. In this case, as with muscarin, atropin had no effect, but digitalin in Ringer's solution often revived it. In short, pilocarpin, though undoubtedly possessing muscle depressant action in large doses, does not seem by any means to be very active in this respect, especially when used in moderate amount. Were the primary stoppage the result of direct cardiac muscle depression, it is impossible to conceive how the muscle could so often overcome the result of this depression while still in actual contact with a strong solution of the drug.

On Gaskell's Electrical Experiment on the Tortoise Heart

Gaskell showed that in the heart of the tortoise, when the auricle was cut away from the sinus and the coronary nerve left intact, the isolated auricle and ventricle preparation would, after a time, beat with its own rhythm, and then stimulation of the right vagus nerve in the neck would diminish the size of the beats of the auricle[1], this showed that the vagus must be active during the period of quiescence, the non-manifestation of that activity being merely due to the want of suitable means of making it visible. Later on, he describes a method by means of which he ascertained that stimulation of the vagus in the neck caused some alteration in the non-beating muscle of the auricle, which was manifested by an electrical change of an opposite sign to that which accompanied contraction of the muscle[2], this change in the quiescent auricle he did not get on stopping the sinus by muscarin directly applied, and from this it is argued that muscarin does not act through the inhibitory nerves.

Under normal conditions the sinus acts as the leader of the heart, the auricular and ventricular beat following in direct sequence to the lead of the sinus. When the heart is inhibited by the application of muscarin to the sinus, standstill is observed on account of the fact

1. *Journal of Physiology*, Vol. IV, p. 85.
2. *Journal of Physiology*, Vol. VIII, p. 406-407.

that the leader is stopped and no active inhibitory action is set up in the auricular or ventricular tissue as long as the drug does not come directly into contact with these parts. If muscarin acts on the nerve endings, then in Gaskell's experiment the sinus would of course stop as the result of local stimulation of the nerve endings in the sinus, but there is no reason why its influence should be apparent by the production of an electrical change in the separated auricle with which it did not come into contact; it merely acted locally at the seat of application, and no change, electrical or otherwise, could be expected in any other part of the heart.

On the other hand, this experiment affords evidence that muscarin does not indirectly influence the auricle by acting on the sinus ganglia or on the vagus fibres passing through the sinus (pre-ganglionic or post-ganglionic). The experiment, however, is quite in accord with the view that muscarin acts on nerve endings.

Heart of Newt

Newts were examined in April and June. In both cases ova were in evidence, and more particularly in the June newts. In April, several hearts were found to respond to faradisation and to pilocarpin, but as a rule it was not possible to prolong inhibition for any considerable period; in general the heart gradually escaped from the effects of stimulation and seemed unaffected by a stronger application of the faradic current or pilocarpin. Some hearts gave no response when acted on by an interrupted current, and in these no pilocarpin effect was obtained. In the June newts it was practically impossible to elicit any trace of inhibition.

Under normal conditions, as proved by the researches of Professor MacWilliam, the heart of the newt is most susceptible to vagus stimulation, very weak excitation causing arrest of whole or part of the heart according to the nerve acted upon; local inhibition is also easily procured as the result of direct local application of the faradic current, the part arrested being inexcitable to direct stimulation. Several features of vagal stimulation peculiar to the newt's heart seem to be reproduced when the heart is arrested by pilocarpin.

Owing to the general unsatisfactory condition of the newt-hearts examined it was difficult to arrive at definite conclusions about some of the points examined, but there was a certain amount of evidence showing the possibility of obtaining local inhibition by careful circumscribed application of the drug ; again, during strong pilocarpin inhibition, there was a distinct diminution in the tendency of the heart to respond to direct stimuli, a response being readily elicited only as the drug effects began to wear off. It is hoped that a fuller investigation of these points may be made later, when the newt-heart becomes more normal with regard to inhibition.

Heart of Salamander

A few experiments performed on the salamander-heart in June elicited the fact that inhibition was but little in evidence at this season. In this heart there was practically a reproduction of what was noted as occurring in the frog-heart during the spring and summer months. In several hearts stimulation of the vagus nerve seemed to cause no change, or if any change was in evidence it generally consisted of slight acceleration of the rhythm. In one heart electrical stimulation had a very slight effect, and this heart was brought to a standstill for about thirty seconds by one drop of a 2 per cent. pilocarpin solution : after this time it began to beat and very quickly regained its former rhythm. Subsequent soaking with 2 per cent. pilocarpin failed to affect it for a considerable time. After fifteen minutes it gradually became weaker and slower, but was beating weakly after one hour : the application of atropin did not help to restore the beat. In another heart faradisation of the vagus trunk gave no result, but direct stimulation of the ventricle caused a temporary stoppage ; this, however, soon passed over, and though stimulation of increased strength was now applied, no change took place in the rate or strength. In this heart pilocarpin was without effect.

A heart in which temporary slowing occurred as the result of direct stimulation to the sinus region, gave no result on the exhibition of pilocarpin. It is interesting to note that all the specimens utilised were distended with ova and obviously approaching the spawning

season. Some similar experiments were done in September and October. In the September experiments the amount of inhibition present was not great, but in general much more distinct than was found in the above hearts in June. In the October experiments inhibition was more in evidence. In the salamander it is likely that inhibition becomes still more marked towards the winter and early spring months.

As in other hearts, it was very apparent that injury to the cardiac walls or cutting of the bigger vessels tended to destroy any little inhibition that might be present, for in no case was inhibition obtained where any serious injury occurred in exposing the heart.

Conclusions

1. In the early months of the year, and especially in the season of increased sexual activity, there is a general diminution of inhibitory power in the frog-heart ; when faradisation of the inhibitory apparatus has little effect, muscarin and pilocarpin also produce but very slight effects ; when vagus stimulation slows the heart, muscarin or pilocarpin will do so also : in short, the effect produced on the heart by small doses of muscarin and pilocarpin is in direct proportion to the influence exercised on the heart by the vagus nerve.

2. Faradisation applied for some time to a heart not possessed of much inhibitory power will tire out the mechanism and the heart will escape ; muscarin or pilocarpin subsequently applied has, in this case, no effect ; consequently, after a heart escapes from the action of pilocarpin, it cannot be arrested by vagal stimulution.

3. Mechanical injury resulting in loss of blood tends to diminish the inhibitory power in even a normal heart, and often destroys it in a case where it was not originally present to any marked extent.

4. Muscarin and pilocarpin in small doses, when dropped on the heart, have no appreciable immediate depressant effect on the cardiac muscle. In very weak hearts, a comparatively small dose may cause weakening, but this takes place gradually and differs from true inhibition in not being helped by atropin ; digitalin tends to increase the force. Many hearts will beat in a strong solution

(*e.g.* 2 per cent to 5 per cent.) of pilocarpin for a long period without much apparent lessening of strength or rate.

5. In the eel-heart there were found great differences in the inhibitory power of the vagus, these differences being most marked in the spring months, when, in some cases, faradisation of the vagus or sinus gives no result. As noted for the frog-heart, in cases where vagus faradisation is ineffective, no result is obtained by the application of muscarin or pilocarpin; the converse of this holds true.

6. Contractile force is similarly affected by pilocarpin and muscarin and by vagus stimulation—diminished or annulled in the auricle while not directly influenced in the ventricle: the mode of recommencement after standstill also corresponds in the two cases—the tendency of the 'interjugular' part of the sinus to initiate the renewal of rhythm being evident in the eel-heart.

7. A condition corresponding somewhat to the antagonism of muscarin and pilocarpin described by Ringer, is sometimes probably the result of a primary excitation of inhibitory nerves by muscarin followed by paralysis of these same nerves on the addition of a pretty large dose of pilocarpin; the same effect in certain hearts can be obtained by using muscarin or pilocarpin alone (frog).

8. Gaskell's muscarin experiment on the tortoise-heart does not exclude the possibility of the drug acting through the nerve endings.

9. In the heart of the newt and salamander, inhibition is absent or markedly diminished at certain periods corresponding roughly to the seasons of sexual activity. Here the same general conditions obtain as were noticed in the frog and eel heart.

10. All the available evidence shows that in different types of the vertebrate heart the local application of muscarin and of pilocarpin reproduces the special results obtained by vagal stimulation in each heart, widely different as they are in different types as regards the parts of the heart acted on, the characters of the action, etc.

11. The above observations strongly support the view that muscarin and pilocarpin in small doses cause arrest of cardiac action

indirectly through excitation of the inhibitory nerve mechanism ('nerve-endings'), and not directly in virtue of their depressant action on the contractile mechanism.

In conclusion, it affords me much pleasure to express my thanks to Professor MacWilliam for much valuable advice and assistance during the course of this investigation.

OBSERVATIONS ON THE ACTION OF BOERRHAVIA DIFFUSA

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Natural Order Nyctagineœa, popularly known as Punarnava in Bengal and Gadha Purna (Hind.).

Distribution.—Widely through Bengal and Chota Nagpur, extensively found during the rainy season.

Description of the plant.—It is a large diffusely-branched herb, with opposite leaves which are faintly undulated at the margin, small and panicled, flowers capitate ; stamens one or two to five ; anther didymous ; ovary oblique and slender ; ovule erect ; stigma peltate ; fruit enclosed in the ovoid ; seed adherent with testa.

There is another plant closely allied to this—it is a red variety, stronger and larger, more branched, lasting longer and bearing red flowers. It resembles the white variety in every particular. The roots of the red plant are bigger, thicker and longer than the white variety.

Popular uses.—It is extensively used for food purposes, specially by the Bengalee ; the whole plant is used as fodder for cattle in the Central Provinces under the idea that it increases the quantity of milk. Illiterate persons use a decoction of the root regularly as a sedative to the urinary passages, specially if there is lessened urinary secretion.

Its antiquity as a drug.—From a very early period its medicinal value was recognised, as may be inferred from its mention in Raj Nirghanta and Bhabaprokash (two very old Sanskrit works on medicine).

Composition.—The whole root and half of the stem were first

turned into a pulpy mass with the help of pestle and mortar. This mass was then macerated with water (twenty times its volume) for about a week and then filtered through a fine rag. The filtered extract was then evaporated to dryness ; the dried mass was treated with dilute sulphuric acid (equal parts of water and H_2SO_4) for a week. The acidulated extract was then filtered, and the filtrate was neutralised with ammonia until it gave a distinct alkaline reaction and until no more precipitate began to fall. The precipitate was allowed to settle, the upper liquid was decanted off and the contents evaporated to dryness. A portion of this was treated with petroleum ether, giving no change.

Another portion was treated with absolute alcohol, which dissolved it entirely in the course of three days ; the alcoholic liquid was concentrated on a vapour heater until only a little of the fluid remained. On the surface of the fluid floated a very minute quantity of black scum, suet-like in appearance and sticking on the hand like gum on touching.

The black scum was removed by decanting and treated separately with ether, in which it was perfectly soluble ; on evaporation of the ether, a greenish-black sticky substance was left. Under the microscope it looked like small oil globules with green colouring matter. This substance is, therefore, probably fatty in nature.

Finally the alcoholic extract was evaporated in an air chamber, when a dry brown powder was left. This powder was soluble in alcohol and satisfied all the tests of alkaloid except that ferric chloride gave only a slight brown precipitate. Under the microscope it appeared as dark amorphous granules. The brown powder was then treated with dilute sulphuric acid and water in equal parts and kept for twenty-four hours so that the whole of the granular mass was dissolved—the amount of dilute sulphuric acid added being about twenty times. This fluid was then evaporated to one-tenth of its volume and allowed to cool, when small needle-shaped crystals began to settle at the bottom ; the whole was then kept in hot air chamber so that extra fluid evaporated and crystals only were left behind.

Physical character of these crystals.—They were small, needle-shaped, brownish-white in appearance when in mass ; taste nearly bland or very faintly bitter ; they were light and voluminous. Under the microscope they appear as long or short needle-shaped crystals, about one-fifth to one-tenth mm. in length. This is evidently the sulphate of the granular body obtained after evaporation of the alcoholic extract. It resembles impure quinine sulphate. The amount extracted was very small, so that the plants were very poor in the amount of the alkaloid. Thus 20 oz. of the original plant treated yielded only 300 mgrms. of the sulphate (about 5 grains) or about ·0005th part of the original plant taken.

The ash of the dried plant yielded only about 15 per cent. of the solid matter ; it was partially soluble in water, and the solution gave tests for chlorides and sulphates of the alkali metals (specially of sodium and potassium), and minute traces of nitrates, and a very faint trace of chlorate.

Thus we see the composition to be (1) a sulphate of a body, alkaloidal in nature ; (2) an oily amorphous mass of the nature of fat (probably) ; (3) sulphates and chlorides and traces of nitrates and chlorates from the ash.

The amount of the alkaloidal body is very small.

Physiological Effects

A guinea-pig, weighing about 500 grammes, was given 200 mgrs. of the sulphate with milk ; it took the drug well and developed no poisonous symptoms. Only change noticed was that the animal passed a very large amount of urine for two days.

As the quantity of the alkaloid obtained was very small in amount, all the experiments were henceforth carried out with a liquid extract of the plant (1 in 10).

The same animal was injected with 4 drachms of the extract at 12 noon, the only symptom it developed was that there was a little excess of urine passed by 4 o'clock ; now another injection of 2 drachms

was given, which was repeated at 6 o'clock; poisonous symptoms began to appear late at night, about seven hours after the last injection; the animal became stupid, took no food that was given to it at night, passed neither stool nor urine, had occasional convulsions and a difficulty in standing. Next day it became perfectly stupid although consciousness was retained; later in the day it became more or less comatose, with frequent convulsions. All the time there was no urine nor any stool. In this state it remained till 5 o'clock in the evening, and died at 6 o'clock in deep coma.

The post mortem examination made next morning showed the following appearances:—

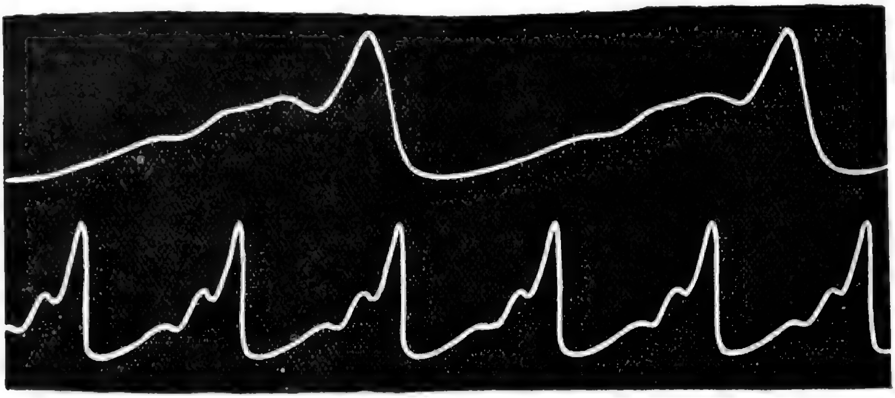
(1) Stoppage of the heart in ventricular systole.
(2) Acute inflammation of the glomeruli of the kidneys, which were acutely congested and almost flesh coloured—more so at the cortical region; on section of the kidneys the glomerular portions stood out prominently as so many reddish dots.
(3) Congestion of the portal area, possibly due to the engorgement of the auricles and right side of the heart.

Microscopical examination of kidney.—Section of kidney showed excessive cell infiltration in and around the glomeruli; the glomeruli were large and standing out prominently amongst other tissues; around the glomeruli there appeared to be a kind of thin fibrinous deposit. The tubules showed practically no change except a little hazy material occupying the lumen of the tubules. Other organs showed practically no change.

Death was probably due to uraemia caused by the stoppage of the excretions from the kidneys.

Effects on isolated heart.—Direct application of the drug on the heart increases the force and frequency of the heart beat; the duration of the systole is increased along with the force of the beat so that all the blood is squeezed out of the ventricle during the contraction and the heart appears paler for a time. In its increasing the force and frequency of the heart beat the drug resembles strychnine; while in increasing the force of systole it resembles digitalis. The following

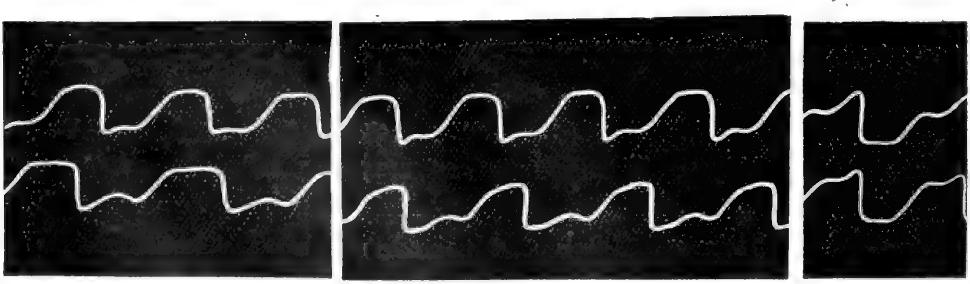
is a tracing of the frog's heart before and after the application of the drug while it was beating forcibly :—



Cardiographic tracing of frog's heart before and after the application of the drug; the upper one represents the normal tracing before the application of the drug; the lower one represents the tracing after the application of the drug; all the while the heart was strong and beating forcibly.

The upper tracing represents the normal beats, while the lower tracing is taken after the application of the drug. In this lower curve the systolic tracing is sharper and more prolonged, indicating the force of the systole, while the downstroke, representing the pause, is about the normal or very slightly longer.

The following tracing was taken while the heart was weak and beating slowly; various doses of the drug were applied—first two drops, then four drops and then treble the dose. Here the upper curve represents the normal, while the lower curve after the application of the drug.



1st Dose Double dose Treble dose

Cardiograph of frog's heart after the slowing of the heart due to weakness.

Here, too, we find the systole more prolonged and sharper, systolic plateau being distinctly longer after the application of the drug. The tracing after the third dose is quite remarkable.

In both cases the tracing represented by the systole is more prolonged and sustained.

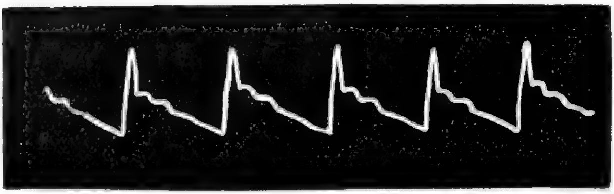
This action of the drug on the heart is probably muscular and not nervous, as may be deduced from the following experiments.

Stimulation of the vagus at the crescent after the application of the drug causes the heart to cease beating, while the removal of the stimulus and re-application of the drug restores the former action of the heart. First, Stannius's ligature after the application of the drug causes the inhibition of the heart, while removal of the ligature and re-application of the drug brings about the contraction of the heart after a little while. Again, application of the drug after double Stannius's ligature brings about one or two contractions of the apex. Thus we see that the nerve (vagus) is unaffected by the drug, and the action exerted by the drug on the heart is probably muscular.

The number of heart beats was markedly increased, and it rose from 76 to 90 in case of one frog; in another from 68 to 81; in a third from 70 to 80, and so on. These phenomena were more marked while the heart was in its wane, and then the frequency in the number of beats was well marked.

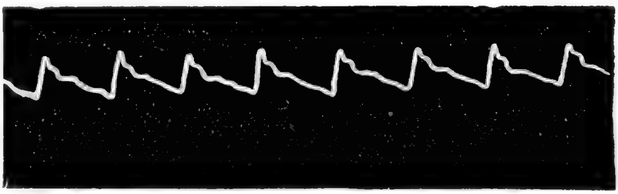
Effects on pulse beat in man.—These facts were also borne out by the condition of the pulse as experimented on human beings. The following tracings indicate the condition of the pulse before and after the application of the drug.

Sphygmographic tracing before the administration of the drug. Pulse beat = 83 per minute; sphygmographic pressure = 92.



Sphygmographic tracing after administration of the drug. Pulse beat = 92 per minute. sphygmographic pressure = 128. Here the upstroke is longer and sharper, while the downstroke is more prolonged and the dicrotic wave is well marked.

Sphygmographic tracing before the administration of the drug. Pulse beat = 82 per minute; sphygmographic pressure = 95.



Sphygmographic tracing after the administration of the drug. Pulse beat = 108 per minute; sphygmographic pressure = 140. Here, too, the dicrotic wave is very well marked, the upstroke longer and sharper.

Similarly, other pulse tracings were taken, and all gave similar results. In all the cases we see that the upstroke due to the expansion of the artery is sharp and long, consequent on the increased force and duration of the systole of the left ventricle. The dicrotic wave is more prominent in all the cases, and that is probably due to the larger amount of blood flowing into the aorta and thereby closing the semi-lunar valves more completely and tightly.

The pulse rate is also quickened—thus :—

	Before		After
Roghu	82	...	108
Gopu	78	...	82
Gokul	83	...	92

The blood pressure is also raised, as experimented by sphygmometer ; thus we have :—

	Before		After
Roghu	95	...	140
Gopu	70	...	120
Gokul	92	...	128
Beshua	85	...	122

This increase in blood pressure is purely cardiac and not peripheral; this was seen by the effect of the drug on the frog's mesentery; it neither dilated the blood vessels nor contracted them. Thus we see that this rise of blood pressure of the arteries is purely cardiac. Hence we conclude that the condition of the pulse exactly coincides with that of the heart and *vice versa.* Consequently from these experiments we draw the following conclusion :—

That the drug increases the force, strength and frequency of the heart beat, increasing at the same time peripheral blood pressure, which is purely cardiac.

The dose of drug used in these experiments in case of human beings was a single dose—4 drachms of the extract—and the results were recorded two hours after the administration.

Experiments on kidney secretion.—It has already been shown that 200 mgrs. of the alkaloidal sulphate caused increased secretion of urine in guinea-pigs. The effects on the urinary secretion in man were tested as follows :—The drug was given in 2-drachm doses of the extract every four hours ; altogether four doses being given during twenty-four hours.

Before the application of the drug.

Name	Quantity of urine in twenty-four hours	Urea	Chloride	Total solid
Gokul	535 c.c.	13 grm.	7·7 grm.	32·6 gr.
Seria	1070	13·7	7·9	35·2
Seionandan.......................	720	13·6	5·2	26·3
Bishua...........................	855	14·2	9·9	28·2

After the application of the drug.

Gokul ....1st day		600 c.c.	6·9 gr.	7·32 gr.	33 gr.
	2nd day	860	15·4	12·88	39·6
	3rd day	960	9·2	18·6	42·7
	4th day	1400	13·6	19	44·1
Seria ....1st day		1110	12·2	10·6	39·4
	2nd day	1200	14·7	15·4	43·5
	3rd	1480	13·2	20·6	46
Seionandan...1st day		1480	12·6	13·1	39·7
	2nd day	2000	14·7	14·28	46·01
	3rd day	2600	15	21·7	49·5
Bishua ......1st day		1092	15·5	12·7	41·7
	2nd day	1209	13·6	15·2	42·1
	3rd day	1600	15·4	22·04	45·4

Now, if we compare the results, we find a general increase in the quantity of urine ; if we further analyse the results we find that the chlorides are proportionately increased as quantity of urine increases ; urea also increases in a few cases, but bears no proportion to the increase of urine. The quantity of total solids, although increased, only maintains the relation with the quantity of urine increased, and, as may be seen, the initial coefficients were not greater, or even in some cases less.

Now these facts exactly coincide with the observations made above ; thus we saw that the injection of the drug in fatal dose caused a state in the kidney, affecting the blood vessels and the glomeruli and causing total cessation of the watery secretion of the kidney ; we also observed that there was increase in the force and strength of the heart beat as well as a rise of blood pressure, which is also evidenced in the arterioles of the kidney, and the result is that the watery part

is thrown out, relieving the blood pressure. Increased and proportional increase in the excretion of chlorides is also suggestive, for a chloride is a salt easily diffusible through the membranes, and hence it is that it shares the same fate as the water, or, in other words, it comes out with the water that is excreted out in excess.

Now increase in the secretion of urea may suggest some increased secretion from the tubules of the kidney ; but the experimental facts are not quite in favour of it. As we have seen in case of guinea-pigs that the tubules showed no sign of affection, it may be possible that they would have been affected had there been sufficient time, but as it is no definite opinion about it can be given ; moreover, the increased secretion of urea is merely comparative and not a real increase.

Such experiments were also conducted by Dr. Harris, of the Medical College Hospital, on clinical cases, in 1901 ; the drug was given indiscriminately to all cases having oedema and kidney affections or ascites, and in almost all cases the urinary secretion, specially the watery part, increased in a marked degree.

Recently the drug was tested in some of the cases in the Hospital; and although the urine increased in every case, the results cannot be given here as the experiments were done so irregularly and perfunctorily. I had the fortune to try the drug in three cases having ascites and oedema, and they all showed increase in the urinary secretion.

Dr. Satis Ch. Banneryu, late House Surgeon and now Assistant Professor of Physiology, also testifies to the increase of urine by the administration of the drug.

Other practitioners of the town also recommend its use in urinary troubles, and testify to its value in increasing the secretion of the urine.

From these data we can infer that the urinary secretion, so far as the watery part is concerned, is invariably increased, that this increase occurs in healthy persons as well as in diseased ones, that the glomeruli are mainly if not solely responsible for the secretion and that the tubules, if affected, are only partially so ; the increase of chlorides is proportional to the increase of the watery part and that the increase of urea is possibly merely comparative.

The drug has been said to have some action on the respiratory system and digestive system, but the experiments on those organs brought about negative results.

Conclusions

1. The active principle is a diuretic chiefly acting on the glomeruli of the kidney through the heart, increasing the beat and strength, and raising the peripheral blood pressure in consequence; on the cells of the tubules it exerts little or no action and, if any, it is only initial and comparative.

2. On respiration it has little or no action, and if it is anything, it is probably due to the fatty principle found in the weeds.

3. On liver the action is principally secondary and in chemical combination with other drugs.

4. On other organs the drug has practically no effect.

From what has been gone through it may be inferred that the drug may be given in any condition of the kidney where there is lessened secretion or where increased secretion of kidney is wanted. Thus it may be given in all renal affections stopping secretion of kidney, in ascites, either from cirrhosis of liver or heart or kidney. As it increases the systole of the heart it may be useful in all stenosed conditions of the valves, as by increasing the force and duration of the systole it can pump all the blood from the heart. Where there is dropsy and ascites due to weakness of the heart or to dilatation of the heart, this medicine, in my opinion, may do extreme good by relieving the circulation through the kidney. In pleurisy and some such affections where there is accumulation of fluid in the cavities, the drug may be useful by increasing the quantity of urine.

A CONTRIBUTION TO THE STUDY OF CALCIUM METABOLISM

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(*Communicated by* PROFESSOR W. A. OSBORNE, *University of Melbourne*)

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INTRODUCTORY

When an animal is deprived of inorganic salts in its food profound constitutional disturbances resulting in death are produced. The salts of the blood must not only be present in sufficient quantity to bring the osmotic pressure of the blood to a constant value but they must also be present in certain definite ratios. Every living cell of the body must be washed by a fluid containing salts of certain mono- and divalent metals in an unvarying ratio otherwise a disturbance in the intracellular ion-proteins (Loeb)[2] or the intracellular colloidal salts (Osborne)[3] is produced. Bearing in mind this necessity for a constant ratio to exist between the various salts of the blood, a number of interesting points are raised when we consider what will be the probable effects of depriving an animal, completely or partially, of salts of one particular metal, say calcium. If the proper ratios are maintained in the blood then the following eventualities present themselves as likely to occur :—

1. The excretion of calcium is checked wholly or partially. During the progress of my research an article appeared by Goitein[4] which disposes of this supposition, for he showed that, if a rabbit

1. Dated at Melbourne, October 7th, 1907.
2. *Dynamics of Living Matter*, New York, 1906.
3. *Journal of Physialogy*, Vol. XXXIV, p. 84, 1906.
4. *Pflüger's Archiv.*, Vol. CXV, p. 118, 1906.

received less than 0·16 grammes of calcium per kilo. per day in its food, there was a steady loss of calcium from the body. Lehmann[1] has also shown that in starvation the calcium excreted exceeds the amount of this substance present in the drinking water taken.

2. The other salts of the blood are reduced *pari passu* by increased excretion. This, however, would entail a considerable fall in the total molecular concentration of the blood and as the living cells of the body and also the red corpuscles are extremely sensitive to osmotic changes this supposition may also be ruled out.

3. The deficiency in the food is made good by certain tissues of the body giving up a portion of their calcium to the blood and so keeping the proper inorganic balance in this fluid. That this would be the most probable contingency may be inferred from a number of facts. Förster[2] who was the first to make observations on the effect of insufficient calcium in the food found that the muscles lost 56 per cent. of their calcium content, whilst the bones also showed a considerable diminution. Voit[3] found that on a calcium-poor diet the bones were more brittle, the skeleton showed a smaller percentage of dry weight than in the normal animal, and that the quantity of calcium in all the organs of the body was more or less diminished.

If such a reservoir exists in the body we might expect that its calcium content would show considerable variations and be influenced by the calcium in the food, *e.g.*, would not only be capable of yielding calcium to the blood but of storing the same if an excess of calcium were absorbed from the alimentary canal. To help the elucidation of these points three series of experiments were conducted. In the first series rabbits were placed on a calcium-poor diet and, after a suitable interval analyses were made of the percentage of total ash as also the percentage of calcium of the blood, in order to see if the ratio of the calcium to the total mineral matter of the blood actually remained

1. Abstract in *Maly's Jahresbericht*, Vol. XXIII, p. 497, 1894.
2. Abstract in *Maly's Jahresbericht*, Vol. III, p. 251, 1874.
3. *Zeit. für Biologie*, Vol. XVI, 1880.

the same as in normal control animals. The bones were also analysed in order to see if the calcium content had diminished relative to the other inorganic ingredients. In the second series metabolism experiments were conducted on myself; a simple but liberal diet was taken with excess not only of calcium but also of nitrogen. Analyses of urine were also made as regards most of its constituents. In the third series advantage was taken of a suitable case of rectal feeding in hospital in which a diet insufficient in protein but comparatively rich in calcium was administered. Analyses were made of the calcium and nitrogen in the enemata administered and in the faeces or wash-out from the bowel.

Chemical Methods Employed

Total nitrogen was determined by the Kjeldahl method; chlorides by the Volhardt method; purin nitrogen by the Walker Hall purinometer; phosphates by titration with uranium acetate standardised against disodic phosphate; total sulphates and ethereal sulphates by the usual method. For determining urea the methods given by Folin[1] were ordinarily followed, but in the experiments with rectal feeding the hypobromite method was considered sufficiently accurate for urea. Sodium and potassium in urine were estimated by the method of Garratt[2]; calcium and magnesium in urine by the method given in Hoppe Seyler's handbook[3]. Calcium in food and faeces was determined by first ashing the material by the method of Neumann and then carrying out the quantitative estimation of calcium in the acid solution.[4] Calcium in blood was determined by drying the latter, obtained from the carotid artery, and ashing with fusion mixture; bone was broken up, dried and also ashed with fusion mixture; in both cases the calcium was estimated in the usual way, namely, as oxide. The total ash of blood was determined by taking 10 c.c. from

1. *American Journal of Physiology*, Vol. XIII, p. 45, 1905.
2. *Journal of Physiology*, Vol. XXVII, p. 507, 1902.
3. Der siebente *Aufl.*, p. 420, Berlin.
4. *Ibid.*, p. 397.

the carotid artery of the anaesthetised animal, placing this in a weighed crucible, evaporating and burning over a low bunsen flame. The residue was then extracted with water, the ashing continued over a blow-pipe flame, and the watery extract added in the usual manner. For determining the total ash of bone, a weighed amount of dried bone was placed in a tared crucible, burnt, and ignited to constant weight over a blow-pipe flame. All reagents employed were tested for impurity but with negative results.

Experimental

I.—*Experiments with Rabbits*

The animals used for the following experiments were the ordinary brown wild rabbits which had been netted, and brought to the laboratory. Young, healthy animals of both sexes were used, care being taken to exclude lactating females, in order that the secretion of milk, which is rich in calcium, might not vitiate the results.

The control animals were fed on a green vegetable diet. This consisted mainly of grass, but lettuces and cabbages were used as well.

The experiment animals were fed on oatmeal, as it has been found that animals on this diet soon suffer from calcium insufficiency. At first the oatmeal was made into a thick paste with distilled water ; but it was found that this caked into a hard mass which was refused by the animals. So the oatmeal was given in the dry form, and a dish of distilled water for drinking was placed in the cage. During the first days of each experiment, a certain amount of green food was also given, until the animal became accustomed to the dry diet. During the later experiments, maize meal was added to the animal's diet of oatmeal and distilled water. To carry out the analyses the animals were anaesthetised with ether. A cannula was put into the carotid artery in the neck, and the animal was bled as long as the blood ran freely. Care was taken not to continue the bleeding so long that fluid would be withdrawn from the tissues and cause an error in the composition of the blood. The blood was collected in a measured vessel ; a certain amount was measured off and used to estimate the

ash of the blood, while the remainder was dried and its calcium-content was determined. After getting the blood in this way, the animal was killed by breaking the neck. The femora and humeri were then cut out, cleaned of muscles and tendinous attachments, pounded up and dried. Their total ash and calcium content were then estimated. An examination of the internal organs was made, but no definite microscopic changes were observed. In some of the animals, a hyperaemia of some of the joints was observed, the synovial membrane and cancellous tissue were engorged, but there was no effusion into the joint-cavity, nor any sign of adhesions in the joint. These changes affected mainly the larger joints, which were always more markedly altered than the smaller joints. These changes were noted in three of the animals fed on calcium-poor diet (one of which died) and in one animal fed on green vegetable diet. In these cases no endocarditis was observed, and the significance of the condition is not known.

In order to obtain a greater amount of calcium in blood, and so, if possible, to diminish the experimental error, in one of the series of experiments two animals were used at the same time in some of the later experiments. Ten c.c. of blood from each were ashed, and the rest of the blood was measured and mixed, dried, and the calcium-content determined.

Rabbit I.—Buck: was anaesthetised and killed on November 19th, 1906, after being brought from the country, where it had been feeding on the natural grasses.

Under ether, carotid artery in the neck cannulised and 48 c.c. of blood withdrawn; of these 8 c.c. were dried and ashed, and 40 c.c. dried and the calcium-content estimated. The femora and humeri were removed, cleaned, smashed up, dried; and the total ash and calcium-content determined.

Results:	Bone, ash	1·0596 g. in 1·8545 g. dried bone, = 57·08 per cent.
	Ca	0.5191 g. in 1·6047 g. dried bone, = 32·35 per cent.
	Blood, ash	0·0502 g. in 8 c.c. = 0·627 g. in 100 c.c.
	Ca	0·0026 g. in 40 c.c. = 0·0065 g. in 100 c.c.

Bone, $\dfrac{\text{Ca}}{\text{Total ash}} = \dfrac{56·6}{100}$ Blood, $\dfrac{\text{Ca}}{\text{Total ash}} = \dfrac{1·04}{100}$

Rabbit II.—Doe: died on November 26th, 1906, after one week on oatmeal and distilled water diet. At the post-mortem examination there was synovial engorgement affecting the large joints (hips, knees and shoulders). The femora and humeri were smashed up and dried.

Results: Bone, ash 1·1243 g. in 1·8339 g. dried bone, = 61·31 per cent.
Ca 0·2178 g. in 0·8679 g. dried bone, = 25·09 per cent.

$$\text{Bone, } \frac{\text{Ca}}{\text{Total ash}} = \frac{41{\cdot}08}{100}$$

Rabbit III.—Doe: killed on November 29th, 1906, after being kept in a hutch since November 19th, 1906, on green vegetable diet. 45 c.c. of blood obtained from carotid artery; of these 10 c.c. dried and ashed, and 35 c.c. dried and their calcium-content estimated. The femora and humeri were removed, smashed up and dried.

Results: Bone, ash 1·8572 g. in 2·8760 g. dried bone, = 64·24 per cent.
Ca 0·3131 g. in 0·8788 g. dried bone, = 35·61 per cent.
Blood, ash 0·0476 g. in 10 c.c., = 0·476 g. in 100 c.c.
Ca 0·0035 g. in 35 c.c., = 0·01 g. in 100 c.c.

$$\text{Bone, } \frac{\text{Ca}}{\text{Total ash}} = \frac{55{\cdot}4}{100} \qquad \text{Blood, } \frac{\text{Ca}}{\text{Total ash}} = \frac{2{\cdot}1}{100}$$

Rabbit IV.—Doe: killed December 7th, 1906, after being fed since November 19th, 1906, on oatmeal and distilled water. 30 c.c. of blood obtained from carotid artery; of these, 10 c.c. were dried and ashed, and 20 c.c. dried and their calcium-content estimated. The femora and humeri were smashed up and dried. Post-mortem: hyperaemia of the cancellous tissue and synovial membrane was observed; most marked in the knees and shoulders, less marked in the elbows and hips, and still less marked in the small joints. No endocarditis.

Results: Bone, ash 0·9619 g. in 2·0749 g. dried bone, = 46·36 per cent.
Ca 0·1810 g. in 0·9015 g. dried bone, = 20·09 per cent.
Blood, ash 0·0663 g. in 10 c.c. blood, = 0·663[1] g. in 100 c.c.
Ca 0·0091 g. in 20 c.c. blood, = 0·0455[1] g. in 100 c.c.

$$\text{Bone, } \frac{\text{Ca}}{\text{Total ash}} = \frac{43{\cdot}3}{100} \qquad \text{Blood, } \frac{\text{Ca}}{\text{Total ash}} = \frac{6{\cdot}8}{100}$$

Rabbit V.—Doe: killed December 20th, 1906; fed since December 8th, 1906, on oatmeal and maize meal and distilled water. 22 c.c. of blood obtained from carotid artery; of these, 10 c.c. were ashed, and 12 c.c. were dried. The femora and humeri were smashed and dried.

1. Some error in the method of analysis undoubtedly occurred in this case.

Results: Bone, ash 1·1771 g. in 2·5805 g. dried bone, = 45·23 per cent.
Ca 0·3396 g. in 1·2874 g. dried bone, = 26·39 per cent.

Blood, ash 0·0525 g. in 10 c.c. blood, = 0·525 g. in 100 c.c.

$$\text{Bone, } \frac{\text{Ca}}{\text{Total ash}} = \frac{58}{100}$$

Rabbits VI and VII.—Killed January 29th, 1907, fed on green grass. 10 c.c. of blood from each was ashed; and 60 c.c. of mixed blood was dried and their calcium-content determined. The femora and humeri were cleaned, smashed up and dried.

Results: Bone, ash 1·5231 g. in 2·2982 g. dried bone, = 66·31 per cent.
Ca 0·3580 g. in 1·2510 g. dried bone, = 28·62 per cent.

Blood, Ca 0·0054 g. in 60 c.c. = 0·0090 g. in 100 c.c.
ash 0·1451 g. in 20 c.c. = 0·7255 g. in 100 c.c.

$$\text{Bone, } \frac{\text{Ca}}{\text{Total ash}} = \frac{43\cdot1}{100} \qquad \text{Blood, } \frac{\text{Ca}}{\text{Total ash}} = \frac{1\cdot2}{100}$$

Rabbits VIII and IX.—Killed March 27th, 1907; fed since January 30th, 1907, on oatmeal and maize meal and distilled water. 10 c.c. of blood from each ashed; 53 c.c. (26-27) of blood dried, and their calcium-content estimated. The femora and humeri were smashed and dried.

One of these animals showed hyperaemia of the synovial membrane of the larger joints.

Results: Bone, ash 1·0485 g. in 2·1306 g. dried bone, = 49·18 per cent.
Ca 0·3059 g. in 1·3966 g. dried bone, = 21·90 per cent.
Blood, Ca 0·0087 g. in 53 c.c., = 0·0164 g. in 100 c.c.
ash 0·1591 g. in 20 c.c., = 0·7955 g. in 100 c.c.

$$\text{Bone, } \frac{\text{Ca}}{\text{Total ash}} = \frac{44\cdot5}{100} \qquad \text{Blood, } \frac{\text{Ca}}{\text{Total ash}} = \frac{2\cdot06}{100}$$

Rabbit X.—Doe: killed March 28th, 1907, after being fed since January 30th, 1907, on oatmeal, maize meal and distilled water. 10 c.c. of blood were ashed; 30 c.c. dried and the calcium-content estimated. The femora and humeri were cleaned, smashed up and dried. Post-mortem showed engorgement of shoulders, hips and knees; no other joints affected; no endocarditis.

Results: Bone, ash 0·8196 g. in 1·6392 g. dry bone, = 50 per cent.
Ca 0·3196 g. in 1·6392 g. dry bone, = 19·49 per cent.
Blood, Ca 0·0046 g. in 30 c.c., = 0·0155 g. in 100 c.c.
ash 0·0833 g. in 10 c.c., = 0·8330 g. in 100 c.c.

$$\text{Bone, } \frac{\text{C}}{\text{Total ash}} = \frac{38\cdot98}{100} \qquad \text{Blood, } \frac{\text{Ca}}{\text{Total ash}} = \frac{1\cdot86}{100}$$

TABLE I.—RABBIT EXPERIMENTS

Rabbit	Feeding	$\frac{\text{Bone Ca}}{\text{Bone ash}}$	$\frac{\text{Blood Ca}}{\text{Blood ash}}$
I	Green grass	$\frac{56.6}{100}$	$\frac{1.04}{100}$
II	Oatmeal	$\frac{41.08}{100}$	—
III	Green grass	$\frac{55.4}{100}$	$\frac{2.1}{100}$
IV	Oatmeal	$\frac{43.3}{100}$	$\frac{6.8}{100}$[1]
V	Oatmeal and maize meal	$\frac{58}{100}$	—
VI and VII	Green grass	$\frac{43.1}{100}$	$\frac{1.2}{100}$
VIII and IX	Oatmeal and maize meal	$\frac{44.5}{100}$	$\frac{2.06}{100}$
X	Oatmeal and maize meal	$\frac{38.98}{100}$	$\frac{1.86}{100}$

It will be seen from the above table that the amount of calcium in the blood varied only between 1 and 2 per cent. of the total ash. On the other hand, considerable variations took place in the calcium content of the bones. Though the results are not quite uniform, it will be seen that the average calcium percentage of the bones of animals fed on oatmeal, and oatmeal with maize meal, is lower than that of the normal control animals fed on green stuff.

II.—*Metabolism in Man*

The experiment was continued for five days. All the estimations were made with undried substances.

Diet.—For lunch weighed amounts of biscuit (thin Captain) and butter were taken, and after the first day a measured quantity of milk was added. For tea and breakfast weighed amounts of oatmeal

1. See previous note.

with a small quantity of salt, were boiled for hours in a double saucepan and a measured quantity of milk was taken with the oatmeal.

The body-weight was taken daily at a regular hour, due precautions being taken to ensure the same clothes being worn : the same weighing machine was used throughout.

The exercise taken consisted of a four mile walk each day.

The work done during the day was the ordinary routine work of the Physiological laboratory.

About eight hours sleep was obtained each night.

The total urine passed in the twenty-four hours was collected, mixed and measured ; the specific gravity was taken and the amount then diluted to the nearest convenient whole number to avoid fractions in multiplication. Samples of this diluted urine were then used for analysis ; a few drops of chloroform were added to prevent decomposition.

The total faeces were collected, weighed, and a sample kept for analysis ; a few drops of formalin being added to prevent the growth of moulds.

As nearly as possible a full analysis of the urine was carried out, but owing to the lack of a suitable colorimeter and the expense of absolute alcohol, the estimation of the creatinin excreted could not be made.

It was intended at the outset to attempt to estimate the complete inorganic metabolism ; but it was found that no suitable method of reasonable accuracy could be obtained to estimate the sodium and potassium when present in small quantities mixed with large quantities of organic matter and phosphates. Therefore, the estimations of sodium and potassium in food and faeces were not made. The calcium was estimated in food, urine and faeces ; since accurate methods can be used for isolating and determining the calcium even when mixed with organic matter.

Table II

Date July, 1906	Weight kilos.	Food Lunch	Food Tea	Food Breakfast	Urine c.c.	Faeces gm.
23	71·8	Biscuit 100 g.	Oatmeal 230 g.	Oatmeal 230 g.	1,200	248
		Butter 20 g.	Salt 20·6 g.	Salt 20·6 g.		
		—	Milk 290 c.c.	Milk 290 c.c.		
24	72·3	Biscuit 121·5 g.	Oatmeal 230 g.	Oatmeal 230 g.	1,620	166
		Butter 20 g.	Salt 16·5 g.	Salt 16·5 g.		
		Milk 400 c.c.	Milk 348 c.c.	Milk 232 c.c.		
25	72·7	Biscuit 119 g.	Oatmeal 230 g.	Oatmeal 230 g.	1,705	115·5
		Butter 19·5 g.	Salt 16·5 g.	Salt 16·5 g.		
		Milk 375 c.c.	Milk 348 c.c.	Milk 238 c.c.		
26	72·8	Biscuit 123 g.	Oatmeal 230 g.	Oatmeal 230 g.	1,815	163·5
		Butter 18·5 g.	Salt 16·5 g.	Salt 16·5 g.		
		Milk 325 c.c.	Milk 348 c.c.	Milk 232 c.c.		
27	72·7	Biscuit 122·5 g.	Oatmeal 230 g.	Oatmeal 230 g.	1,975	98
		Butter 21 g.	Salt 16·5 g.	Salt 16·5 g.		
		Milk 315 c.c.	Milk 348 c.c.	Milk 232 c.c.		

Table III

Date	Calcium			Nitrogen		
July, 1906	Food	Urine	Faeces	Food	Urine	Faeces
23	1·2640 g.	0·1083 g.	—	18·37 g.	12·14 g.	7·23 g.
24	1·7373 g.	0·1687 g.	1·4956 g.	21·08 g.	13·55 g.	2·85 g.
25	1·6912 g.	0·1651 g.	1·2532 g.	20·91 g.	13·60 g.	1·94 g.
26	1·6428 g.	0·1665 g.	1·3420 g.	20·63 g.	14·46 g.	2·95 g.
27	1·6311 g.	0·1680 g.	0·9276 g.	20·58 g.	14·18 g.	1·76 g.

From the above tables it will be seen that during the course of the experiment the body was not in nitrogenous equilibrium, but was retaining nitrogen. The weight of the body, it will be observed, had increased.

Intake	101·47 g. N
Output	84·66 g. N
Balance	16·81 g. N

This would represent a storing of about 109 grammes of proteid during the five days.

The calcium also shews a gain to the body.

Intake	6·7024 g. Ca
Output	5·6867 g. Ca
Balance	1·0157 g. Ca

The excretion of Ca by the urine shows a remarkable constancy of about 0·166 gramme during the last four days of the observations, while the faecal excretion shows a gradual diminution.

TABLE IV—URINE

Date	Amount and sp. gr.	Total N in grs.	Purin N in grs.	NH_3 in c.c. $\frac{N}{10}NH_3$	Urea in grs.	Chlorides as Cl_2 in grs.	P_2O_5 in grs.
23	1200 1023	12·13	0·117	450	15·12	10·3	1·79
24	1620 1031	13·55	0·156	288	10·90	14·9	2·58
25	1705 1024	13·6	0·312	420	18·78	16·5	2·34
26	1815 1021	14·46	0·166	484	24·35	19·5	2·60
27	1975 1020	14·18	0·300	424	17·57	21·6	2·83

TABLE IV—*Continued*

Date	Total SO_3 in grs.	Ethereal SO_3 in grs.	Ca in grs.	Mg in grs.	Na in grs.	K in grs.
23	2·03	0.20	0·1083	0·1093	5·3181	3·351
24	2·26	0·17	0·1687	0·1216	8·7483	3·283
25	2·42	0·17	0·1651	0·1083	8·5568	3·1003
26	2·54	0·18	0·1665	0·1126	10·690	3·168
27	2·40	0·12	0·1680	0·1215	12·2848	2·912

This table shows a gradual but marked increase from day to day of the chlorides and sodium, with a less marked rise in the excretion of phosphates, and magnesium. On the other hand the calcium remained almost constant.

III.—*Metabolism in Rectal Alimentation*

This series of observations was carried out on a patient at the Melbourne Hospital, and I am indebted to Dr. Howard for permission to investigate the case.

M. G., *aet.* 26, was admitted to the Melbourne Hospital on the 28th August, 1906, suffering from the symptoms of gastric ulcer. Rectal feeding was carried out from the 26th August until the 18th September, the only thing allowed by the mouth was plain filtered water.

The food administered, by four-hourly nutrient enemata, consisted in solutions of plasmon in water, starch in 0·7 per cent. salt solution, and white of egg in water. These were used in varying amounts during the greater part of the experiment. It was found that the amount of plasmon calculated as necessary to keep up the nitrogenous equilibrium could not be got into solution even in the total amount of water used in the twenty-four hours, so that it became necessary to reduce the amount of plasmon injected. During the last three days various nutrient enemata were tried, with the object of increasing the calcium intake.

The patient's rectum and sigmoid colon were washed out each morning at 8 a.m., and the return, together with the faeces, if any, was mixed, measured, and a sample reserved for analysis. The total twenty-four hours' urine was collected, measured, and samples taken for analysis.

The weight of the patient was taken at 11 a.m. on the first two days, but removal to the scales caused such abdominal discomfort that weighing had to be discontinued.

On the 1st September, the patient commenced to menstruate, and this function continued for four days, during which the nutrients were continued but the urine and faeces were not analysed.

In the samples of urine and faeces obtained the total nitrogen and total calcium were estimated, and the urea of the urine was approximately determined by the hypobromite method.

Table V—Rectal Feeding

Date	Weight	Food				Water		Urine	Bowel Return		
Aug., 1906		Plasmon	Starch	Egg-white	5 per salt sol.	By bowel	By mouth		Faeces	Washout	Total
	Kilos	Grs.	Grs.	C.c.	C.c.	C.c.	C.c.	C.c.		C.c.	C.c.
25	—	—	—	—	—	—	—	300	—	—	—
26	55·9	86	11·5	114	170	910	340	1070	—	824	824
27	55·5	86	11·5	85	170	1160	170	1140	—	454	454
28	—	86	11·5	71	170	1160	200	1350	350	460	810
29	—	86	11·5	57	225	1160	280	2205	—	895	895
30	—	56	11·5	57	170	1130	280	2500	—	1050	1050
31	—	56	11·5	85	200	990	250	1595	—	700	700
Sept.											
1-4	On same diet.										
5	—	56	11·5	58	225	1075	225	1335	—	1400	1400
6	—	56	11·5	64	170	1075	250	1330	—	1150	1150
7	—	56	11·5	57	170	1075	140	1400	—	525	525
8	—	56	11·5	57	170	1075	200	1555	—	630	630
9	—	56	11·5	57	170	1075	250	1035	225	1035	1260
10	—	56	11·5	57	170	1075	425	1770	—	575	575
11	—	56	11·5	57	170	1075	310	1650	200	1230	1430
12	—	56	11·5	57	170	1130	170	1350	130	525	655
13	—	56	11·5	57	170	1075	250	1430	—	765	765
14	—	56	11·5	57	170	1075	250	1450	—	1005	1005
15	—	56	11·5	57	170	1075	170	1180	—	835	835
16	50	Peptonised milk 850 c.c.					280	870	230	1180	1410
17	—	Pepd. mk. 570c., stch. 23g. sal. 340 c.c.					250	925	—	1220	1220
18	—	Stch. 23g., 4 eggs, saline 990 c.c.					250	1035	—	845	845

TABLE V (*continued*)—RECTAL FEEDING

Date	Calcium			Nitrogen			
Aug., 1906	Food gr.	Urine gr.	Faeces gr.	Food gr.	Urine gr.	Faeces gr.	Urea gr.
25	—	0·1978	—	—	6·88	—	14·12
26	1·5530	0·2040	0·1146	12·47	14·51	0·72	—
27	1·5492	0·1186	0·0363	11·92	9·14	0·23	18·27
28	1·5473	0·2133	0·2366	11·72	12·36	2·29	22·14
29	1·5455	0·2813	0·5156	11·40	17·32	3·13	32·7
30	1·0090	0·2859	0·1841	7·80	15·57	5·73	29·9
31	1·0127	0·2474	0·0478	8·32	11·16	1·76	20·9
Sept.							
5	1·0090	—	—	7·80	—	3·52	—
6	1·0099	0·2588	0·3506	7·93	9·36	2·28	17·2
7	1·0090	0·2652	0·0309	7·80	10·15	0·42	19·5
8	1·0090	0·2629	0·1896	7·80	10·97	0·94	19·8
9	1·0090	0·2434	0·8910	7·80	8·40	4·02	15·8
10	1·0090	0·3008	0·2556	7·80	9·84	1·27	18·8
11	1·0090	0·2798	0·1638	7·80	7·60	3·20	14·3
12	1·0090	0·3086	0·1571	7·80	6·40	1·31	11·7
13	1·0090	0·2941	0·0953	7·80	6·54	0·78	11·0
14	1·0090	0·2853	0·5919	7·80	7·67	4·22	14·0
15	1·0090	0·1961	0·2925	7·80	6·14	1·77	12·0
16	0·9936	0·2313	0·2429	4·74	8·30	2·36	13·5
17	0·6663	0·2033	0·1539	3·18	5·39	1·39	10·1
18	0·3082	0·2582	0·0136	4·29	5·56	0·53	10·7

The preceding table may conveniently be divided into three periods:—

1. From 26th to 31st August inclusive, during which the patient was fed on varying amounts of plasmon, starch and egg-white. On the first days, probably not all the plasmon weighed out was actually injected, since the solution formed a thick jelly which could only with difficulty be made to run through the nutrient tube.

Intake ...	... 8·2167 g. Ca ...	63·63 g. N_2
Output ...	... 2·4855 g. Ca ...	93·92 g. N_2
Balance ...	...+ 5·7312 g. Ca ...	−30·29 g. N_2

It is interesting to note that the urine excreted on the day the patient had no food contained 0·19 gramme Ca, while the amount

excreted during the rest of the experiment varied between 0·11 and 0·30 gramme Ca.

2. During the next period, Sept. 6th to 15th inclusive, the patient had settled down and the results are more valuable, since the nursing staff had become accustomed to the routine necessary, and further, there was now no difficulty with the plasmon solution gelatinising in the nutrient tube during administration.

Intake ...	... 10·0909 g. Ca	...	78·13 g. N_2
Output ...	... 5·7133 g. Ca	...	103·28 g. N_2
Balance ...	...+ 4·3776 g. Ca	...	−25·15 g. N_2

3. During the third period a varied diet gave the results:

Intake ...	... 1·9681 g. Ca	...	12·21 g. N_2
Output ...	... 1·1032 g. Ca	...	24·53 g. N_2
Balance ...	...+0·8649 g. Ca	...	−12·32 g. N_2

Over the whole period the results are:

Intake ...	... 20·2757 g. Ca	...	153·97 g. N_2
Output ...	... 9·3020 g. Ca	...	221·73 g. N_2
Balance ...	...+ 10·9737 g. Ca	...	−67·76 g. N_2

The nitrogen shews a steady leak from the body. This shews that it was impossible to give by the bowel sufficient, even of a highly nitrogenous substance like plasmon, to keep the body in nitrogen equilibrium.

The urinary calcium shews a remarkable constancy throughout the experiment.

Discussion and Summary

In the experiments in which rabbits were fed on oatmeal and maize meal, a diet which admittedly leads to calcium starvation, the ratio of the calcium of the blood to the total ash of the blood remained much the same as that found in the normal animal. That is to say, the blood underwent no loss of calcium relative to the other salts in the time allotted to the experiment—a result which one might anticipate from the immense importance of the salt ratios of the

blood. The ratio of calcium to the total mineral matter in the bones was, however, inconstant, and shewed fairly wide fluctuations even in the normal animal. The bones can, without doubt, act as reservoirs of calcium (and possibly of magnesium). That they lose calcium when the animal is placed on a calcium-poor diet has been proved so conclusively by others, that I have not thought it necessary to carry out confirmatory experiments. My results, however, tend to show that the bones can lose calcium relatively to the other salts, that is, by a selective autolysis and not by an autolysis of bone in mass. The experiments on my own metabolism show that calcium can be readily stored during nitrogen retention. More interesting, however, are the experiments with rectal feeding, where calcium was stored despite a continuous drainage of nitrogen from the body. In this latter case, as the proteid absorbed from the food was insufficient, the muscles and glands must have been diminishing in bulk, and yet calcium was retained. This fact rather points to the bones as the seat, in this case, of calcium storage.

In the experiments on myself and in those with rectal feeding, it will be noticed that, with a fixed diet, the urinary calcium varied but slightly, and the variations such as they were ran parallel with the total amounts of urine excreted. This result is not remarkable if we assume that the kidney, in order to lighten its work against osmotic pressure, allows a fraction of each of the salts of the blood to escape into the urine. The greater the volume of the urine, therefore, the greater the mass of salts eliminated.

Results of quantitative determinations of certain urine constituents are given as metabolic data of some importance.

I have to thank Professor Osborne for the use of his laboratory and for his invaluable advice and kindly criticism.

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ON THE EQUILIBRIUM BETWEEN THE CELL AND ITS ENVIRONMENT IN REGARD TO SOLUBLE CONSTITUENTS, WITH SPECIAL REFERENCE TO THE OSMOTIC EQUILIBRIUM OF THE RED BLOOD CORPUSCLE[1]

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Since the pioneer experiments of Sidney Ringer[2] first demonstrated the absolute necessity of certain saline constituents in the circulating fluid supplied to the isolated contracting heart, in order that the heart muscle should be enabled to carry on in a normal fashion its physiological functions, a host of observers have been fascinated by the study of the reaction of the living cell to changes in the soluble inorganic constituents of its environment.

Nor can any subject be regarded as of higher practical importance either in regard to the rational study of biological function, or of practical medicine which ought to form the applied science of such a study.

It is becoming ever more clearly recognised that the various drugs which show a specific action in different diseases, produce their results by the action of a specific inorganic or organic ion added to the medium which surrounds the cell, and possessing, by virtue of its constitution and the constitution of the cell affected, special affinity for that cell. So that one class of cell is most affected by one ion and another class by a different ion.

1. The expenses of this research have been chiefly defrayed from a grant made by the Government Grant Committee of the Royal Society.

2. *Journ. of Physiology*, Vols. III, IV, VI, etc. See Schäfer's *Text-book of Physiology*, Vol. II, p. 225.

It is on such a basis that physical chemistry has given a rational explanation of why, for example, all mercury salts produce the same specific action in syphilis, the result being due to the free mercury ion, and not being affected by the anion of the salt used except in so far as this quantitatively alters the degree of ionization and hence the concentration of mercury ion. Similarly, the ferric ion, in all iron salts, stimulates the production of erythrocytes in anaemia. So too, quinine, and the alkaloids generally, furnish a basic ion affecting specific cells of the organism, or of pathogenic foreign organisms present in it, for which at different stages they possess special affinities.

It is on this specific selective affinity of different cells of the organism or of the cells of parasites of the organism, for ions or molecules in solution in the medium bathing such cells, whereby these cells selectively adsorb or combine with such ions so changing the activities of the adsorbing cells, that the rational experimental science of therapeutics of the future must be based.

As an instance of how different organic ions attack and combine, or become adsorbed, by different cells, we may instance the observation of Whitley[1] in regard to the action of phenol-phthalëin and di-methyl-amido-azo-benzol on the eggs of *Echinus esculentus* (sea urchin) and *Pleuronectes platessa* (plaice) respectively. A trace of phenol-phthalëin rapidly killed the echinus eggs but had no injurious action on the plaice eggs; while, conversely, a trace of di-methyl-amido-azo-benzol rapidly killed plaice eggs, but had absolutely no injurious effect on echinus eggs.

If we suppose for a moment that the plaice eggs and echinus eggs represented two parasitic organisms infesting an animal which was to be treated, then di-methyl-amido-azo-benzol would have been a specific drug for the one, and phenol-phthalëin for the other, providing that these two bodies did not injuriously affect the higher animal acting as host.

It may be added that this holds not only for drugs, but for the toxins of disease which usually produce their effects on account of their specific adsorption by the body cells.

1. *Proc. Roy. Soc.*, B Vol. LXXVII, p. 137, 1906.

In view of the abundant evidence from physiology, pathology, and therapeutics of the ability of the cell to combine, or enter into adsorption with ions from its bathing fluid, and that the amount of adsorption or combination taking place varies in degree for each ion, and often in a highly specific manner from one type of cell to another; it is somewhat surprising what a very passive rôle has been assigned to the cell in this process by most workers in biology and physiology in considering the normal adaptation of the cell to its environing medium.

It is one of the oldest and best established experimental findings of biology, that the inorganic constituents of the interior of the cell differ widely from those of the plasma or intercellular fluid by which the cell is bathed. The cell salts are rich in potassium and phosphates, while the plasma is exceedingly poor in concentration of these ions, and richer in sodium and chlorine ions. Since the cell receives all its nutrition, both inorganic and organic, from the outer fluid, the existence of such a difference points most powerfully to the cell contents possessing a special affinity, or adsorbing power, for the potassium and phosphates on account of which it takes them up from the intercellular fluid, although they are only present there at such low concentration; and, on the other hand, to the fact that its constitution gives it no adsorbing or combining power for the sodium, so that although this ion is present in the intercellular fluid in many times higher concentration than the potassium ion it is not taken up by the cell, which has no attachment for it and no facility for holding it.[1]

This is not, however, the accepted view, according to which the cell is regarded as acting quite passively as to what it shall take up and what reject in the way of ions from its enveloping fluid. The whole exchange is supposed to be regulated for the cell by an inert membrane by which it is enclosed, and which, like a prison wall, keeps the potassium and phosphate ions within while it equally

1. See Moore, *Recent Advances in Bio-Chemistry*, edited by Leonard Hill, Arnold, London, 1906, p. 149 *et seq.*

prevents sodium ions from entering.[1] The membrane theory takes no trouble to explain how prisoners are introduced within the prison, yet introduced in some way they must be, for as growth proceeds, cells multiply, and there is no diminution in the number of imprisoned ions in each.

In the present paper we desire to suggest (i) that the *qualitative* differences between the electrolytes of the cell and the electrolytes of its environment, are due, not to the presence of an impermeable wall, but to the specific affinities of the cell protoplasm for certain electrolytes or ions, whereby it combines with or adsorbs them, and (ii) that in regard to *quantitative* relations there is maintained an *equilibrium* in regard to electrolytes between the cell and its environment, but this equilibrium does not necessarily consist in an equality or isotonism in regard to total osmotic pressure, and in practically all cases departs in some degree from equality.

Considering first the qualitative composition of the salts of cell and plasma, we may pass over the fact that the existence of a limiting membrane has not been proven in the vast majority of cases, and assuming the existence of such an impermeable membrane, discuss whether it is capable of explaining the profound differences in composition of cell salts and plasma salts which have been found by all previous observers, and are shown in our own determinations later in this paper.

Can the cell become vastly richer in potassium and phosphates and poorer in chlorides and sodium than its surrounding medium by the action of a membrane ?

The answer to this question is perfectly clear, the only membrane which could produce such an effect would be one which was permeable in one direction and not in the other, in other words, a Maxwell's demon would be required to open a trap-door in one direction and close it in the other.

Such a membrane, with pores or valves open in one direction

1. See Hamburger. *Osmotisches Druck und Ionenlehre in den medicinischen Wissenschaften*, Bände I u. III, where a full account is given of the literature of the subject and the experimental work by various authors, which is supposed to establish the membrane and permeability hypothesis.

and closed in the reverse direction, is unknown in inorganic nature, and has not been shown to exist in the case of living cells.[1]

On the other hand, it has been clearly established that colloids possess strongly marked affinities for combining with crystalloids, or for adsorbing them, and this condition is all that is requisite for explaining the difference in qualitative composition between cell salts and plasma salts.

Let us suppose that the cell starts with a complete equality, both qualitative and quantitative, as regards its crystalloids, with the surrounding fluid (plasma) ; then the known existing condition of richness in potassium and phosphates and poverty in sodium and chlorides obtaining within the cell can be realized by taking into account that the cell protoplasm combines or fixes in some chemical or physical way the potassium and phosphatic ions, while the plasma similarly holds the sodium and chlorine ions.

There are many examples known of such adsorption or combination between colloids and crystalloid substances when present together in common solution.

Perhaps the simplest and best known example which can be taken to illustrate such an action of adsorption in heaping up concentration in one portion of a heterogeneous system (such as cell and nutrient medium form) and depressing it in another portion of the system, is the taking up of oxygen by the haemoglobin of the red blood corpuscle.

Here, just as is the case with potassium and phosphatic ions, under normal conditions the concentration of oxygen in the red blood corpuscle is many times that which it has in the plasma. Circumstances of oxygen pressure can also be chosen which will *apparently* prove that the red blood corpuscle is impermeable to oxygen, notwithstanding this large amount of oxygen inside, on similar lines to those

1. It may be pointed out, since this point has escaped the attention of previous writers on the subject, that a passive valved membrane will not serve the purpose of obtaining an increased concentration or pressure on one side. The valves, to open, must either have energy supplied from outside, or must take momentum from the molecules passing through. In other words, the mere presence of valves permeable in one direction cannot explain increased concentration of the permeable substance on one side by passage from a more dilute solution. Any such concentration, in other words, requires a supply of energy.

which have wrongly been taken as proving that the red blood corpuscle is impermeable to potassium ions.

If, for example, pressures of oxygen be taken between 200 mm. and 1,000 mm., and the blood corpuscles and plasma be shaken up with the oxygen until equilibrium is attained, and then the concentration of oxygen in corpuscles and plasma determined. It will be found that while the amount of oxygen in the plasma is almost proportional to the pressures, the amount of oxygen in the corpuscles is very little different at 1,000 mm. to what it is at 200 mm. pressure.

The result, as we know well, is due to the fact that haemoglobin and oxygen combine or become adsorbed with each other and that the adsorption is almost complete at about 120 mm. pressure. At this point nearly all the large amount taken in by the haemoglobin in physical or chemical union is already within the corpuscle, and the further rise in pressure of oxygen only causes to be taken in the small amount capable of existing in uncombined or unadsorbed condition.

It is obvious, therefore, that the true explanation is adsorption and not impermeability of a membrane in the case of oxygen and haemoglobin.

Yet this is exactly the kind of experimental proof upon which the view is based, that although the red blood corpuscle is very rich in potassium yet it is impermeable to that ion. Because no amount of extra potassium, which can be shown by chemical analysis, enters the red blood corpuscle when it is washed with normal saline made with potassium chloride, or no *appreciable* amount of potassium passes out of it when it is contrariwise washed in a saline made of pure sodium chloride, the deduction is drawn that the red blood corpuscle is impermeable to potassium.

From what has been said above in the case of haemoglobin and oxygen, however, it follows clearly that such a deduction can only be drawn provided the concentration of potassium has been so reduced that the pressure of dissociation of the compound or adsorpate of potassium and cell constituents has been passed ; for until this has been done the cell will not appreciably lose potassium, and at pressures above the dissociation value the cell will not appreciably take up

more than its normal amount. Further, if this dissociation pressure be a low one, the determination of small amounts of potassium leaving the cell will be a most difficult one, for the potassium will have to be determined in presence of large excesses of sodium chloride or other indifferent saline used to wash the corpuscles, which must be washed in isotonic solution or otherwise will break up. Also, it must be remembered that the amount of potassium coming out at each wash, with a saline of sodium chloride only, will be very small, since the potassium will only diffuse out until its concentration is at a level somewhat lower than the low level of its concentration in the natural serum, and at each successive wash the concentration in the sodium chloride saline will be lower than in the preceding wash.

A consideration of the low concentration of potassium ion in the normal plasma or serum shows at once that the dissociation pressure of potassium and cell contents must be very low (or in other words, that the combination between cell contents and potassium is a fairly stable one), because it is at this low potassium ion pressure existing in the plasma that the red blood corpuscle and the tissue cell become saturated with their potassium content, and accordingly the dissociation pressure must lie below this low value.

Accordingly it is clear that the exchanges of potassium between cell and plasma, especially when one remembers the poorness of quantitative methods for determination of potassium in presence of excess of sodium, may well be such as to fall within the limits of experimental error in direct chemical analysis.

Fortunately, there are more delicate physiological methods which clearly show that when the amount of potassium in the plasma is increased there is an uptake by the tissue cells, shewing that here there is no impermeability due to a membrane, and that the apparent impermeability is due to a protective action brought about by the cell contents possessing a specific adsorptive power.

Before passing to this physiological proof attention may, however, be drawn to the fact, which never seems to have been appreciated or have had attention drawn to it, of how low the concentration of oxygen is in the plasma when the red blood corpuscle begins to part

freely with its oxygen, and, what particularly concerns us here, of how impossible it would be to estimate this oxygen if we had to depend, as we have in the case of potassium, upon estimations of percentages in the plasma itself.

The partial pressure of oxygen at which appreciable dissociation of oxy-haemoglobin commences can safely be taken as lying below 150 mm., which is about the partial pressure in the atmosphere. Now the coefficient of solubility of oxygen in plasma is about the same as in water, and for the purposes of this calculation may be taken as 0·02. It hence follows that with a partial pressure in the air of 150 mm., the osmotic pressure of the dissolved oxygen in the plasma is only $150 \times 0{\cdot}02$ mm. = 3 mm. This exceptionally low pressure, or rather about two-thirds of it, that is 2 mm. of mercury, is the *real* dissociation pressure of oxy-haemoglobin, and not the 150 mm. obtaining in the air mixture to which the blood is subjected. The name of *osmotic* dissociation pressure might perhaps be suggested for it, to prevent confusion with the partial dissociation pressure in the air to which it is of course proportional.

Now it is this osmotic dissociation pressure which we must take into account in considering the percentage by weight of oxygen in the plasma. When the calculation[1] is made it is found that with an oxygen partial pressure of 150 mm. in the air in contact with the blood, the percentage by weight of oxygen in the plasma is about 0·0005 per cent., that is about half a milligram of oxygen in 100 c.c. of plasma.

Now, if we take it that the potassium in the red blood corpuscle is no more firmly held than the oxygen, so that its appreciable dissociation commenced at about 3 mm. of osmotic pressure, this would correspond to a concentration of K_2O in the plasma of 0·5 mg. per 100 c.c., which is a quantity far outside the limits of chemical analysis in such a solution, and could only be determined if the potassium could be pumped off as a gas like the oxygen.

1. The calculation is as follows:—22,330 c.c. of oxygen, under normal conditions of temperature and pressure, weigh 32 grammes, what will 100 c.c., at 3 mm. pressure weigh $= \frac{32 \times 3 \times 100}{760 \times 22{,}330} = 0{\cdot}00057$. Corrections to body temperature which have not been introduced in the calculation, further reduce the amount to 0·00049, that is to very approximately half a milligram of oxygen in every 100 c.c. of plasma.

If we turn now to the physiological tests in the active tissues, we find that the permeability of the cells for oxygen and for potassium can be shown by exact parity of reasoning in the two cases.

Although the cells do not take up an additional amount of oxygen at two atmospheres of pure oxygen obvious to chemical analysis, as compared to what they take up under ordinary atmospheric conditions, of one-fifth of an atmosphere of partial pressure of oxygen, the physiological effects due to the increased oxygen pressure are profound. At about two atmospheres of oxygen pressure the nerve cells are upset in their oxygen metabolism and the animal dies in convulsions. The pressure of oxygen is so great that the combination between tissue cell and oxygen becomes a permanent fixed one, instead of an unstable one fluctuating in labile equilibrium and being made and unmade as the osmotic pressure of dissolved oxygen varies around the dissociation value of about 2 mm. The entire oxygen exchange so delicately balanced is upset and the cell is as much asphyxiated as if it were deprived of oxygen altogether, or were bound in fast combination with such a gas as carbon-monoxide. In fact, by another method, oxygen exchanges have been made impossible.

Exactly similar results are obtained in the case of the potassium ion which prove unmistakably that this ion passes into or out of the cell, causing increased or decreased potassium ion pressure in the cell when the concentration of that ion in the circulating fluid is raised or lowered.

This is most clearly shewn, perhaps, by the changes in beat of the isolated heart perfused by salines of different composition, as discovered by Ringer. When there is no potassium ion present, or too low a concentration, the automatic rhythmic beat soon stops in a characteristic way; when the low amount of 1 in 10,000 of potassium chloride is added then the beat becomes quite normal, and as the potassium concentration is increased beyond this optimum concentration the beat becomes characteristically irregular and abnormal, and at a slightly higher concentration the heart again ceases to functionate.

The behaviour is exactly as in deficiency or excess of oxygen,

and might be almost described as potassium asphyxiation, with diminution or excess.

Similar proof is given by administration of excess of potassium salts to the animal as a whole, as shewn by the profound depressant effect upon the nervous system of doses which would show to chemical analysis no appreciable increase in potassium ion concentration in either plasma or tissue cells.

In physiological action, then, we possess a test infinitely more delicate than chemical analysis, and the test proves conclusively the permeability of the cell for ions such as potassium and the phosphates. Chemical analyses of cell contents and nutrient fluid give also results which show specific adsorptive powers for the different inorganic constituents.

This specific power of adsorption, which is moreover not equal for different types of cell but varies in a characteristic manner, furnishes an explanation of many physiological and pharmacological phenomena, and does not occur merely in a few isolated instances but in the manifold reactions in which the living cell carries on its wide commerce of exchange in health and disease.

The point made out above, also, that exchange can only occur normally around about a certain optimum of concentration, cannot be too firmly grasped, for excess leads to stoppage of exchange equally with deficiency.

As some instances of this labile equilibrium between the cell and its nutritive constituents having for its chemical basis unstable chemical combinations or adsorpates possessing definite low limits at which they associate or dissociate, there may be mentioned, also, the organic foodstuffs, where there is evidence, always increasing, of combination in both blood and tissues between proteid on the one hand and carbohydrate or fat on the other. There exists at present little doubt as to a loose chemical combination in blood and tissue between proteid and carbohydrate. Further, in the case of the liver, it has been demonstrated that normal liver shewing little or no fat under the microscope may, on extraction, yield as much fat as a pathological liver microscopically shewn to be laden with fat globules. It is

obvious here that the fat in the normal liver must be in some combined form with the protoplasm which renders it homogeneous with the protoplasm and invisible under the microscope. Again, perfectly clear serum may easily contain over one per cent. of fatty extractives which, unless combined in some fashion, most probably with the serum proteids, would yield an obvious, opaque, milky emulsion quite different from the clear serum in which it is contained.

The same unstable, easily dissociated compounds are known to be formed in the case of constituents added from without, or formed by disease. Thus, for example, the stilling action of anaesthetics upon cell activity has been shewn to be due to the limitation of cell activity by the formation of such unstable combinations or adsorpates between cell proteid and anaesthetic, which persist only so long as the osmotic pressure is kept above a certain level, and dissociate and so leave the cell once more active when the pressure of anaesthetic falls again in the course of free respiration.[1]

Similar compounds or adsorpates have had to be assumed, and have now in many cases been proven, for toxins and similar bodies with cells, and for toxin and antitoxin with each other. Here the selective adsorptive power of special cells for special products above referred to, becomes peculiarly evident.

So also in the case of active selective drugs; the action is in each case due to a more or less unstable combination or adsorpate between drug and tissue cell or some special constituent of some definite type of cells. As examples may here be mentioned that of the bromine ion on the central nervous system, and of the nitrite ion, adrenalin, strychnine, or atropin on special nerve cells or nerve endings.

In blood corpuscles and in tissue cells we have, therefore, to deal, not with an impermeable membrane, but with a cell substance possessing specific adsorptive powers, and it is the low solubility in *free* solution in the cell contents as compared with the relatively larger power of taking up in adsorbed or combined condition which gives rise to an apparent impermeability, which is shown not to exist (i) by the presence in the cell of the apparently impermeable con-

1. Moore and Roaf, *Proc. Roy. Soc.*, Vol. LXXIII, p. 382, 1904; also *ibid.*, B, Vol. LXXVII, p. 86, 1905.

stituent in large quantity, and (ii) by the conspicuous physiological effects obtained by increasing or diminishing the osmotic pressure of the apparently impermeable constituent, and so limiting the play of dissociation and association on the part of the cell.

Turning now from qualitative considerations of the equilibrium between the cell and its external medium the plasma, to considerations of the *total* osmotic equilibrium of pressure between cell and plasma, as shewn by depression of freezing point in both cases, we find that the necessary condition is an *equilibrium* of total pressure between cell and outer fluid, and this equilibrium need not necessarily mean an *absolute equality* of osmotic pressure within and without.

It is quite clear in many cases that the cells can exist and functionate in a perfectly normal physiological manner when they are not in a condition of equality in regard to total osmotic pressure with the fluids which bathe them. Thus a salivary gland cell in secretion is in contact on the one side with plasma or lymph and on the other side with the secreted saliva possessing an osmotic pressure much lower than that of the lymph; it is, therefore, perfectly obvious that the osmotic pressure of the cell contents cannot be equal both to that of the lymph and that of the saliva, which are entirely different from each other in value. Similarly, in the case of the secreting cell of the uriniferous tubule, on the one side is lymph and on the other urine with a very varying osmotic pressure as shown by the depression of freezing point it possesses, yet the varying osmotic pressure of the urine never injures the tubule cell, although the osmotic pressure of urine and cell contents never can be in a state of equality. A third example is the columnar cell of the intestinal villus, which has most widely varying pressures on its intestinal side and manages to maintain in its living condition an equilibrium with these, and absorbs fluids of very varying osmotic concentration. In fact, it is the exception and not the rule that the living cell in the body must be in contact with fluids of equal osmotic concentration to its own cell contents. An equilibrium with regard to osmotic contents must exist between cell and bathing fluid, but the cell possesses an adaptability within certain limits, which vary with the type of cell, and the equilibrium

need not consist in an equality of osmotic pressure within and without the cell.

The usual inference that there is an equality of osmotic pressure is based on the plasmolysis experiments in plant cells, and on changes in volume of animal cells, especially the red blood corpuscles, on altering the osmotic concentration of artificial outside bathing fluids.

Now, such experiments are of value in shewing the osmotic pressure with which cells are in *equilibrium* at different volumes of the cell, but they do not demonstrate equality of pressure within and without, or prove that the cell, normally, has an *equality* of pressure with its environing medium under natural conditions; nor can the cell be regarded as a perfectly inert membrane quite free to move and accommodate itself without resistance to all changes in osmotic pressure in the outer medium.

It is further assumed in such experiments that the salts inside the cell and those placed in the medium without are *perfectly* impermeable to the cell, and as has been pointed out above, this is a most dangerous assumption.

It is well to analyse what is the fundamental thing being measured in such experiments on change of volume of cells placed in solutions of varying 'tonicity,' or osmotic pressure.

It is quite clear that the cell will not change in volume if the tendency to a flow of water is equal in the two directions. Hence, the two things pitted against each other in any such experiment, are really two rates of diffusion of water, one inwards and one outwards, and a constant volume, therefore, means an equality in these two rates of diffusion. This, however, may mean something quite different to equality of osmotic pressure within and without, and it is only in the limiting case of *complete* or perfect impermeability for all soluble constituents that equality of rates of diffusion, which the experiment shows, can be taken to mean equality of osmotic pressures. If a dissolved substance passes in and out without any resistance, then it cannot exert any pressure, and will lead to no diffusion of water and have no effect on cell volume. If it cannot pass at all, it will exert its full osmotic pressure when the cell has come to rest, and will exert

its full influence on the diffusion of water and on cell volume. Between these two extremes a partial influence will be exerted; and apart from adsorption, and allowing full time for equilibrium to be attained, then equilibrium will only be reached when the concentrations of free unadsorbed substance are equal on both sides of the membrane for each individual soluble component of the system of dissolved substances.

The plasmolytic method, as a machine or apparatus of physical chemistry for determining isotonicity of two given external fluids, each of which possesses an equally low permeability to the cell, is perfectly legitimate, so long as the assumptions made in the experiment are carefully borne in mind. These are that the permeability must be low for both substances and, theoretically, that the rate of permeability should either be equal in the two cases or so small as to be negligible during the period of the experiment, and secondly, if there is any permeability, as there nearly always must be, that the observations be taken at the same time interval from the commencement.

Given that these conditions are fairly well satisfied, then the plasmolysis method may be a good working method for determining approximate equality of osmotic pressure in different solutions.

It is quite another matter, however, to assume from such experiments that there is an *equality* of osmotic pressure between the cell contents and the environing medium of the cell.

In the case of the vegetable cell, turgidity of the cells and pressure of the contents against the cell wall would be impossible by osmotic action if the effective portion of the osmotic concentration of the outer sap were not less than the effective portion of the osmotic concentration of the cell sap within. When plasmolysis occurs, these two effective portions have become equal, leaving out of account any resilience of the cell contents.[1] But the experiments say nothing as to equality in osmotic concentration of sap within and without, and the effective factor in turgidity is the difference in the two. The effect of slow permeability must also be borne in mind, as well

1. See Drabble and Scott, *Bio-Chemical Journal*, Vol. II, p. 221, 1906.

as cell resilience, which is an important factor in many types even of vegetable cell.

In most types of animal cell the resilience becomes a most important factor, and enables the cell safely to bear great variations in the osmotic pressure of fluids in contact with it. Here the minute size of the cells requires careful consideration, since, given that the dimensions are sufficiently minute, a most delicate structure can support a very high pressure. For example, a very thin-walled capillary glass tube, if its bore be sufficiently small, can bear a pressure of several atmospheres without rupturing, which would at once burst even a thick-walled glass tube of wide diameter. Hence, in the case of a tissue cell of fixed dimensions a very considerable difference of osmotic pressure may be borne without any appreciable change in volume.[1]

Accordingly, we must be slow to argue from observations of changes in volume of cells subjected to treatment with salines of varying concentration, that there exists equality of osmotic pressure at these different volumes within and without the cell.

It seems somewhat strange that it has not occurred to any previous workers on the subject to determine freezing points of both serum and separated blood corpuscles and to contrast such determinations with each other; and further to allow corpuscles to come into equilibrium, as regards osmotic exchange, with salines of different concentration, then separate corpuscles from salines by centrifuging, and determine freezing points in both.

This method gives a direct experimental answer to the enquiry as to whether the osmotic pressures within and without are always equal, or whether there is an equilibrium at each pressure but not necessarily an equality or isotonicity.

In the present experiments we have carried out such measurements, amongst others, and have obtained interesting results which show that neither in the normal condition of natural serum and

1. This result is due to thickness of cell or tube wall, when very small dimensions are reached, being very great relatively to the thickness of wall in wider tubes.

untreated corpuscles, nor in the case of corpuscles brought into equilibrium with salines of different strength is there absolute equality, but rather an equilibrium which varies with the concentration.

It may be argued that the small but constant differences in depression of freezing point which we have observed between natural serum and corpuscles may be due to unequal changes in dissociation in the two cases between body temperature and the freezing point at which, of course, the freezing point determinations have to be made. This we freely admit, but at the same time the freezing point method is the only one available at present for determinations of osmotic pressure in serum and corpuscles, and is the one which has been hitherto relied upon for the supposed proof that there exists equality of pressure between intercellular fluid and cells or, in the present particular case, between blood serum and erythrocytes. We submit that if freezing point determinations are to be quoted in proof of isotonicity, then freezing point of serum and erythrocyte must be shewn to be the same within the limits of experimental error.

In the whole extensive literature of cryoscopic measurements on blood we have only been able to find one instance in which the freezing point of the separated corpuscles has been taken alongside of that of the serum. This measurement was made by Prof. G. N. Stewart[1]; the Δ found for whole blood was 0·628, that for the corpuscles was 0·597, and the author marks the latter with a note of interrogation, evidently taking the difference for an experimental error and pursuing the subject no farther.

We have found a constant difference in this direction throughout all our experiments, viz., that the depression of freezing point of the serum is always somewhat greater (0·02 to 0·03) than that of the erythrocytes, or in other words, that, at any rate at the freezing point, the osmotic concentration of the serum is greater than that within the red blood corpuscle.

When the corpuscles are shaken up with salines of different osmotic concentration, allowed to settle, centrifuged off, and then

1. *Journ. Physiol.*, Vol. XXIV, p. 129, 1899.

cryoscopic determinations made in (*a*) corpuscles and (*b*) supernatent saline, these differences become greatly exaggerated. The corpuscles do not come into equality of osmotic concentration with the outer saline, but preserve a varying pressure of their own, which stands in equilibrium with the outer pressure but is not equal to it.

We have also performed other series of experiments which bear in upon the question before us, of the relationship of inorganic constituents of cells and intercellular fluids, and how these salts are related by adsorption or otherwise to the organic constituents.

One method we have utilised is that of dialysis carried out in comparative experiments in serum and corpuscles. If the dissimilar constitution and composition of the electrolytes of (*a*) serum and (*b*) erythrocyte were due to an impermeable wall, then we should expect that when we disrupt that wall and allow cell contents to escape as, for example, by dialysis against distilled water, the salts previously held in prison by the wall would escape and pass readily into the dialysate, and then we should expect that determinations of osmotic concentrations in the two dialysates would give us at least approximately equal results. If, on the other hand, the electrolytes of the cell are held in adsorption, or some form of chemical combination by the organic cell contents, then we should expect that such adsorption might continue when both organic substance and electrolyte quit the cell as a result of dialysis; and as the organic substance cannot pass the dialysing membrane, neither can the electrolyte which is adsorbed with it. Hence, in this case, the Δ of the dialysate of the corpuscles should be much less than that of the serum. We are here assuming, of course, that the pressure of electrolyte does not fall sufficiently low in the process of dialysis to cause any considerable dissociation of electrolyte and organic cell constituent. This, however, is shown by theoretical considerations given earlier in the paper to be an improbable result, as the osmotic pressure of the electrolyte necessary to cause almost complete adsorption is very low, and hence, when dialysis occurs, osmotic pressure of sufficient value will be very soon restored by the diffusion of a very small amount from the adsorpate of organic constituent and electrolyte.

The results of our diffusion experiments very strongly support the view of an adsorpate between the organic constituents of the erythrocyte and the electrolytes contained therein, rather than the action of any membrane.

Similar results are obtained by repeated freezing and thawing, which lake the corpuscles, the freezing point remaining practically constant under such conditions, although the conductivity alters on account of removal of mechanical resistance.

In addition, we have conducted incineration of serum and corpuscles, and made chemical determinations of chlorine and phosphoric acid, in order, by our own experiments, to make certain of the very different composition of the two ashes shewn by all the older experiments. Further, we have made cryoscopic and conductivity experiments with ashes, dialysates, and original sera and corpuscles, which have clearly demonstrated to us that there is a labile osmotic equilibrium between serum and the cell, which is never characterised by absolute osmotic equality, but shows adsorption by the cell in all cases, and leads accordingly to different disposals of electrolytes and other soluble substances within and without the cell.

It is remarkable that in such an adsorbed form the electrolytes of the cell still have their due effect in producing osmotic pressure, as shewn by the cryoscopic method. Although attached in some form to the protein they still have a full effect in depressing the freezing point. There is, hence, a degree of freedom of the attached ions in adsorption which is lost in ordinary chemical combination, and this, to our minds, is one of the most fundamental differences between such adsorptions and chemical combination. For it is clear, when the relative percentages of salt and protein and relative molecular weights are considered, that there must be several ions in combination with each colloidal protein molecule, yet each of these has its full effect on freezing point as if it were free.

It is otherwise in regard to electrical conductivity, because here the ionic velocity is altered by the attached protein molecule which must be bodily moved with the ions in the electric field. Undoubtedly a part of the very high resistance of the corpuscles is

due, as shewn by Stewart and Oker Blom, to the mechanical effect of the want of homogenity of the fluid in the electric field[1]. This higher resistance, however, disappears only in part when the corpuscles are laked and the field made practically homogeneous save for the ghosts of the corpuscles, and the conductivity of the laked solution is still much lower than that of a dialysate reduced by evaporation to an equal volume, or of the ash after incineration and accompanying removal of haemoglobin.

Thus we have found, in confirmation of Stewart, that the conductivity of corpuscles is only one-fourteenth to one-nineteenth of that of serum ; on laking by freezing and thawing alternately, the conductivity rises to about one-sixth of that of serum ; dialysis brings conductivity up to one-half to one-third of the conductivity of the serum dialysate, and the ash of serum and of corpuscles have nearly the same conductivity.

This still high resistance after laking we attribute to the attachment of haemoglobin to the electrolytic ions, which is further confirmed by the fact that haemoglobin, as shewn by Gamgee, does move in the field during electrolysis.

This independence of ions as regards osmotic pressure (freezing point), and dependence on protein regarding ionic velocity (conductivity) is remarkable, and is probably connected with that extreme degree of labile equilibrium which is everywhere characteristic of the living cell and its energy exchanges.

The experiments on (A) fresh corpuscles and serum, (B) laking, (C) effects of foreign salines, (D) dialysis, (E) incineration, (F) estimation of chlorides and phosphates, and (G) conductivity, were usually made on the same samples of blood so as to admit of comparison with one another, but for clearness of description are placed below in different sections.

1. We have ourselves carried out conductivity experiments with sand and saline, and saline mixed with undissolved starch granules, which completely confirm Oker Blom's theoretical work on the subject.—*Arch. f. d. ges. Physiol.*, Vol. LXXXI, p. 167, 1900.

A.—On the Relative Freezing Points of (*a*) Corpuscles and (*b*) Serum in Fresh Untreated Blood

The blood was whipped as it came from the animal,[1] carefully collected and centrifuged as rapidly and completely as possible. Then serum and corpuscles were pipetted apart into dry vessels, and freezing points were determined as rapidly as possible. The same material was used for the conductivity experiments (Section G).

The value of Δ given is in each case the mean of at least three closely concordant freezing point readings.

No. of Experiment	Serum Δ	Corpuscles Δ
Sample 1	0·607° C.	0·577° C.
Sample 2	0·539° C.	0·519° C.
Sample 3	0·591° C.	0·573° C
Sample 4	0·586° C.	0·570° C.

Thus, the Δ of the corpuscles lies 0·02 to 0·03 lower than that of the serum, this would correspond to a difference of osmotic pressure of approximately 200 to 300 mm. of mercury pressure.

B.—On the Freezing Points (*a*) of Serum and (*b*) of Corpuscles after Laking the latter by Repeatedly Freezing Solid and Thawing

Here, no appreciable change in freezing point occurs, as might be expected were crystalloids set free by rupture of a membrane wall.

	Serum Δ		Corpuscles Δ	
	Freezing solid		Freezing solid	
	Before	After	Before	After
Sample 1	0·607	0·613	0·577	0·571
Sample 2	0·539	0·548	0·519	0·520

1. The blood of the pig was used for all the experiments given.

C.—On the Equilibrium Between Blood Corpuscles and Salines of Varying Concentration

In these experiments three strengths of saline were used, one of which was made very hypertonic, the second nearly isotonic, as shown by its freezing point, and the third hypertonic to the natural serum of the blood corpuscles used. The concentrations of the three solutions used were in the first experiment in the ratios of three, two and one, the more dilute being made from the strongest by diluting in this ratio with distilled water.

The corpuscles, after thorough centrifuging, were pipetted off and mixed with an equal volume, in each case, of the three strengths of the saline solution.

With the view of afterwards testing for potassium, and, if possible, estimating the relative amount of potassium and sodium coming out of the corpuscles by weighing mixed chlorides and determining percentage of chlorine, we used, as our saline medium, calcium chloride instead of sodium chloride. On account of other substances not chlorides, such as phosphates, dialysing out from the corpuscles, the percentages of sodium and potassium were so indeterminate that we could place no confidence in our actual figures by this indirect method of estimating potassium. We satisfied ourselves, however, by precipitation with platinic chloride and cobalt hexa-nitrite, that potassium was present in the calcium chloride saline after mixing with the calcium chloride and centrifuging.

When the centrifuged-off corpuscles had been placed in each of three centrifuge tubes with an equal volume of the three calcium chloride solutions of the three different strengths, they were thoroughly mixed, shaken up several times, and left for an interval of sixteen hours. If the small size of the red corpuscle is considered, it is evident that the process of equalization by diffusion must have been complete and equilibrium established by the end of this period.

The Δ for the serum (Sample 5) in the first of these experiments was 0·551° C., that of the strongest calcium chloride solution was 0·983, and, therefore, assuming no appreciable change in dissociation

from dilution, the calculated Δ of the other two solutions would be 0·647 and 0·323, so that No. 1 was strongly hypertonic, No. 2 slightly hypertonic, and No. 3 strongly hypotonic.

For the second experiment the blood of Sample 3, as used above, was taken, and the Δ determinations were made directly for each of the three calcium chloride solutions, which stood in the concentrations of approximately 2·03 per cent., 1·25 per cent., and 1 per cent.; the lowering of freezing point in each of these solutions before admixture with the corpuscles was found to be 0·919° C., 0·574° C., and 0·465° C., so that No. 1 was strongly hypertonic, No. 2 almost exactly isotonic, and No. 3 strongly hypotonic.

The solutions, after admixture with an equal volume of corpuscles, were left as before, being often shaken up, and then allowed to stand over night.

In the third experiment (Serum Sample 4), on exactly similar lines, three salines of barium chloride, showing Δ's of 0·605° C., 0·308° C., and 0·255° C., were used, instead of calcium chloride solutions. The weakest concentration gave rise to a considerable amount of laking, but the corpuscles still showed, even here, a lesser Δ. As in all the other experiments of the series, no laking was seen in No. 1 of this experiment, and only very little in No. 2.

Throughout the series of experiments it is seen that the osmotic pressure of corpuscles remains much below that of the saline, and this whether the saline is hyper- or hypotonic to the original natural serum of the corpuscles used for the experiments.

EXPERIMENT I ($CaCl_2$)

No. of solution	Original solution Δ	Saline after admixture and centrifuging Δ	Corpuscles after admixture and centrifuging Δ	Difference in Saline Δ and Corpuscle Δ
No. 1	0·983	0·847	0·788	0·059
No. 2	0·647 (calculated)	0·627	0·563	0·064
No. 3	0·323 (calculated)	0·409	0·362	0·047

EXPERIMENT II ($CaCl_2$)

No. 1	0·919	0·828	0·748	0·080
No. 2	0·574	0·598	0·561	0·037
No. 3	0·465	0·622	0·505	0·117

EXPERIMENT III ($BaCl_2$)

No. 1	0·605	0·615	0·581	0·034
No. 2	0·308	0·428	0·399	0·029
No. 3	0·255	0·396	0·375	0·021

D.—ON THE FREEZING POINTS OF (*a*) CORPUSCLES AND (*b*) SERUM AFTER DIALYSIS AGAINST DISTILLED WATER

The object of these experiments has been pointed out above.

In carrying out the experiments, a known volume of corpuscles or serum respectively, usually 50 c.c., was taken, placed in a wide piece of sausage-shaped dialysing tube of parchment paper; this was immersed in a wide-mouthed bottle containing a measured amount of distilled water, usually 450 c.c. All possible precautions were used by sterilizing bottle, dialysis tube, and water against putrefactive change, and dialysis was allowed to proceed for forty-eight hours, the dialysing tube being held in position by clipping it between bottle neck and bottle stopper when placing the glass stopper in the bottle.

After completion of the period of dialysis, the outer fluid was carefully collected in a graduated vessel, and measured. It was then evaporated down to such an aliquot part that it corresponded to the concentrations of the serum or corpuscles originally taken. Suppose, for example, the original amount of serum in the sausage tube was 50 c.c., and in the bottle outside there was 450 c.c. of distilled water, then it is obvious that the serum, when equilibrium was complete, would be diluted tenfold; hence, if at the end, 440 c.c. were found in the water bottle, this was evaporated carefully down to 44 c.c. before its freezing point was taken. Similar procedure exactly was used for the corpuscle dialysate to be compared alongside of the serum.

It was found that the corpuscle dialysate had a much less Δ than the serum dialysate, shewing the stronger adsorption between the organic matter of the corpuscle and the electrolytes of the corpuscle.

Results of Dialysis

	Serum Δ		Corpuscle Δ	
	Before dialysis	After dialysis	Before dialysis	After dialysis
Experiment I (Sample I)	0·607	0·562	0·577	0·232
Experiment II (Sample II)	0·539	0·568	0·519	0·262

E.—Freezing Point of the Ash after Incineration of (*a*) Serum and (*b*) Red Blood Corpuscles respectively

	Serum Δ Incineration		Corpuscle Δ Incineration	
	Before	After	Before	After
Sample I	0·607	0·450	0·577	0·357
Sample II	0·539	0·469	0·519	0·362

It is to be noticed that Δ is decreased after incineration for both serum and corpuscles ; but that the two depressions are much closer than after dialysis where the adsorpate between protein and crystalloids in the case of the corpuscles keeps the crystalloids from passing the dialysing membrane.

F.—Percentage of Chlorides and Phosphates in (*a*) Serum and (*b*) Corpuscles respectively

The relative percentages of chlorides and of phosphates in serum and corpuscles were determined (*a*) after dialysis and (*b*) after incineration by the usual volumetric methods, with the following results.

	Serum percentages		Corpuscles percentages	
	Cl	P_2O_5	Cl	P_2O_5
Dialysis	0·3657	0·0197	0·1331	0·0329
Incineration	0·3373	0·0219	0·1704	0·1708

The results confirm the freezing point determinations, and show that in the case of the corpuscles the phosphates are held back from dialysis by some form of combination or adsorption with the protein;

the chlorides seem to be held in much looser combination, and in part dialyse through, but even here the figure is still much lower in the dialysate (0·1331 as against 0·1704) instead of being somewhat higher as in the serum due to slight volatilization during incineration with such a large excess of organic matter.

G.—ELECTROLYTIC CONDUCTIVITY OF SERUM AND OF CORPUSCLES RESPECTIVELY: (*a*) IN FRESH CONDITION, (*b*) AFTER LAKING BY FREEZING AND THAWING, (*c*) AFTER DIALYSIS AND REDUCTION TO ORIGINAL VOLUME, (*d*) AFTER INCINERATION AND MAKING UP TO ORIGINAL VOLUME

The conductivity was, in each case, determined in the usual manner at 40° C., by Kohlrausch's method, and the specific conductivity calculated.

The figures given, in order to save decimals, are sp. conductivity multiplied by 10^5.

	Treatment to which subjected	Sample I Serum	Sample I Corpuscles	Sample II Serum	Sample II Corpuscles
1.	Fresh... ...	1705	95	1519	109
2.	Frozen Solid (Corpuscles laked)	1602	310	1468	237
3.	Dialysed ...	1843	891	1623	754
4.	Incinerated ...	1608	1677	1697	1655

The figures support the conclusions of the other sections, and the changes in the conductivity of the corpuscles are most interesting. The first increase on laking is probably mechanical, due to removal of the resistance interposed by the want of homogenity caused by the presence of the whole corpuscles. The second increase with dialysis is due to the detachment from the protein of the greater part of the less firmly held chlorides, but the phosphates are here lost on account of their stronger attachment and to their being left behind in the dialysis tube.

Finally, in the incinerated corpuscles and serum both chlorides and phosphates are free, and the conductivity of the corpuscle crystalloids now becomes practically equal to that of the serum crystalloids.

Conclusions

1. The membrane theory fails to explain (*a*) the difference in composition of the electrolytes within and without the cell, (*b*) the physiological effects of perfusion or irrigation of cells by media defective or excessive in certain electrolytes normally present in the cell, (*c*) the selective uptake, accompanied by physiological effects, of certain soluble substances by different cells, such as food constituents, drugs, anaesthetics, and toxins.

2. These phenomena receive a simple explanation, on the basis of the formation of adsorpates or chemical combinations between cell protein (or protoplasm) and other constituents.

3. The cell when functionating normally exists in a labile or mobile equilibrium with such constituents, and is capable of undergoing reversible processes of association or dissociation with such constituents.

4. For each constituent there exists a certain range of osmotic pressure for that constituent, within which partial association and dissociation is possible, and it is within this range alone that labile exchanges are possible. Thus there is a minimal limit of starvation and a maximal limit of plethora for each indispensable constituent beyond which exchange becomes impossible in sufficient amount to meet the requirements of cellular life, and when these limits are passed in either direction, the chemical exchanges become embarrassed, and finally cell activities cease in a form of asphyxiation in a wide sense, showing similar effects at both ends of the range.

Anaesthetics, toxins, and drugs probably produce their effects by causing, when in sufficient concentration, adsorpates or compounds, which upset the cell's metabolism on account of their stability at the given osmotic pressures.

5. The osmotic concentration leading to formation of sufficiently stable adsorpates varies from substance to substance, and in many cases, as notably in that of oxygen, the osmotic concentration at the dissociation range is very low.

6. Cells normally exist and functionate in contact with fluids such as secretions, which are not isotonic with the cell contents.

7. There is not absolute isotonicity between the erythrocytes of blood and serum, the depression of freezing point for serum being always slightly greater than that for the corpuscles.

8. On laking the erythrocytes, the freezing point does not appreciably change.

9. The differences in freezing point are much exaggerated when the corpuscles are brought into equilibrium with either hypotonic or hypertonic saline solutions, and the rule holds that the osmotic concentration of the saline outside is always higher (as in the case of natural serum and corpuscles) than that of the corpuscles, whether the saline was originally hypotonic or hypertonic.

10. Dialysis also demonstrates an adsorpate between the protein and the electrolytes of the erythrocyte, since the depression of freezing point for corpuscular dialysate is much less than that for serum dialysate.

11. The results of dialysis and incineration, and determination of chlorides and phosphates, considered together, show that the adsorpate between protein and phosphates persists at a much lower osmotic concentration than that between protein and chlorides.

12. Determinations of electrical conductivity of corpuscles and of serum, of laked corpuscles and of serum, of dialysed corpuscles and dialysed serum, and of incinerations of corpuscles and of serum have been carried out which shew, (i) that in the natural condition of corpuscles and serum the conductivity of the former is only one-fourteenth to one-seventeenth of that of the latter, (ii) that this difference is partially mechanical, the ratio being reduced to one-fifth to one-sixth on mere laking (confirmation of Stewart's results), (iii) that the ratio is further reduced to about one-half on dialysis, due to setting free of crystalloids from colloids, although, in the case of the corpuscles, the greater part of the phosphates are still held back in adsorption or chemical combination with the protein, and (iv) that finally, on incineration, the conductivity of corpuscles and serum crystalloids becomes practically equal in value.

PROSECRETIN IN RELATION TO DIABETES MELLITUS

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The treatment of diabetes mellitus with secretin is based primarily upon the assumption that the normal stimulus to pancreatic secretion —secretin—is deficient or absent, owing to the lack of prosecretin in the intestinal mucous membrane of diabetic patients. With a view to ascertaining whether this assumption was justifiable, Beddard and I examined the duodenum of several cases of diabetes. Our observations, which were recorded in a former paper, showed that prosecretin was absent, or almost absent, from the duodenal mucous membrane of five out of six cases of diabetes. It seemed desirable to examine a larger number of cases before attempting an explanation of the facts, and the observations made on several other diabetic patients are here described.

Methods

The duodenum and upper part of the small intestine were obtained from the diabetic patients as soon as possible after death; the mucous membrane was scraped off, and an acid extract made, as described by Bayliss and Starling. The activity of the extract was tested by intravenous injection into cats anaesthetised with chloroform and ether, and having a cannula in the pancreatic duct. In each experiment the activity of the extract was compared with that obtained by injecting secretin prepared from normal animals, or from non-diabetic patients. The blood pressure was usually recorded, and the depressor effect observed.

Results

Previous observations have shewn that prosecretin is always present in the intestinal mucous membrane of non-diabetic patients. The following are the results yielded by the diabetic cases.

Case	Sex, age, and cause of death	Clinical duration of disease	Interval between death and preparation of extract	Flow of pancreatic juice produced by extract
I.	Boy, aged 17: diabetic coma	1 year	24 hours	Very scanty flow
II.	Man, aged 21: diabetes and phthisis	About 18 months	12 hours	Fair flow
III.	Man, aged 53: diabetic gangrene	—	8 hours	Good flow
IV.	Man, aged 45: diabetic coma	18 months	30 hours	Fair flow
V.	Woman, aged 42: diabetic coma	5 months	24 hours	Good flow

Case I.—A boy, aged 17, was admitted to St. Bartholomew's Hospital, October 3rd, 1906, for diabetes. Sugar was first observed in his urine in November, 1905.

On admission the patient was very wasted, his urine contained abundance of sugar (300 grammes for twenty-four hours) but no diacetic acid. Two days later he became comatose, and 'air-hunger' was noticed; death occurred early on October 6th.

Post mortem: The pancreas appeared to be normal; the other viscera showed no noteworthy alterations.

The duodenal mucous membrane was obtained and extracted twenty-four hours after death. The extract was injected into a cat anaesthetised with chloroform and ether, and a scanty flow of pancreatic juice was produced.

Case II.—A man, aged 21, was admitted to Guy's Hospital in May, 1906, for thirst and wasting. These symptoms had been noticed for about a year.

On admission, the patient was extremely wasted; his urine contained much sugar, and gave an intense reaction with ferric chloride. He remained in hospital for some months, during which the daily output of sugar varied from 200 to 300 grammes. Death took place on October 9th, 1906, from acute tuberculosis.

The *post mortem* examination was made twelve hours after death. Both lungs showed extensive acute tuberculosis. The pancreas was normal both macroscopically and microscopically.

An extract of the duodenal mucous membrane was tested on a cat anaesthetised with chloroform and ether, and a fair flow of pancreatic juice resulted.

Case III.—A man, aged 53, was admitted to hospital on September 15th, 1906, for gangrene of the right foot.

On admission, his urine was found to contain much sugar. On October 3rd, the right leg was amputated above the knee; death occurred on October 9th, 1906.

Post mortem: The aorta was atheromatous, the heart wall fatty, the kidneys and liver large, but not definitely fibrosed; the pancreas was tough and fibrous.

The duodenal mucous membrane was obtained and tested eight hours after death; the extract, when injected into a cat anaesthetised with chloroform and ether, produced a good flow of pancreatic juice.

Case IV.—A man, aged 45, was admitted to Guy's Hospital in March, 1906, for thirst, polyuria and wasting; he had been losing weight for nearly a year.

On admission, the patient was found to be very wasted; his breath smelt strongly of acetone; his urine contained acetone, diacetic acid, and a moderate quantity of sugar. During his stay in the hospital he passed daily from 70 to 100 grammes of sugar; on one occasion he showed signs of incipient coma. He was treated with secretin by the mouth, but it had no effect on his output of sugar.

Some months later he died, outside the hospital, from diabetic coma. The intestinal mucous membrane was obtained thirty hours later, and an extract was at once made and examined; when injected into a cat it produced a fair flow of pancreatic juice.

Case V.—A woman, aged 42, began to suffer from pruritus and thirst in October, 1906, and in November, sugar and diacetic acid were found in her urine.

She was admitted to the Metropolitan Hospital, under the care of Dr. Langdon Brown, on February 8th, 1907, and placed on a moderately restricted diet. Two days later she became drowsy, and 'air-hunger' was noticed; she soon became completely comatose, and died on February 11th.

The duodenal mucous membrane was obtained twenty-four hours after death. A boiled acid extract, injected into a cat, yielded a good flow of pancreatic juice.

The following protocol shows the amount of pancreatic juice secreted as a result of injecting the various duodenal extracts. As far as could be judged by their depressant action on the blood-pressure, the extracts (with the exception of the cat's secretin) were approximately of the same concentration.

The first four observations were made on the same cat, and are, therefore, more strictly comparable than the last two observations.

Injection	Blood-pressure	Flow of pancreatic juice
5 c.c. Extract from normal cat ...	140 fell to 65 mm. Hg ...	1·6 c.c. juice
10 c.c. " " Case I	140 " 90 " ...	1 drop
10 c.c. " " Case II	130 " 75 " ...	0·4 c.c. juice
10 c.c. " " Case III	145 " 65 " ...	1·0 c.c.
10 c.c " " Case IV	120 " 50 " ...	0·5 c.c.
10 c.c. " " Case V	—	1·6 c.c.
5 c.c. " " Non-diabetic case	120 fell to 60 mm. Hg ...	0·75 c.c.

I am indebted to Professor Starling for permitting me to publish, in a footnote, the particulars of two other cases of diabetes; in each case an acid extract of the duodenal mucous membrane contained secretin.

NOTE.—*Case I.*—Diabetes and phthisis. *Post mortem:* The pancreas was microscopically normal. An acid extract of the mucous membrane contained abundance of secretin.

Case II.—A diabetic patient, operated on for an ischio-rectal abscess, died three days later from diabetic coma.

Post mortem: The pancreas was large and fibrous; sections showed degeneration of the alveoli—the cells being small and free from granules—rather like a pancreas after ligature of the duct. The duodenal extract contained secretin.

Conclusions

In all the cases of diabetes described above, a boiled acid extract of the duodenal mucous membrane contained secretin; with one exception, the activity of the extracts was almost or quite as great as that of extracts from the duodenum of non-diabetic patients.

These cases, taken together with those previously recorded by Beddard and myself, show that prosecretin is more often present than absent in diabetes, and it may be doubted whether the absence of prosecretin has any causal relation to diabetes. It is quite possible, of course, that, as Moore suggests, prosecretin is deficient in *some* diabetic patients during life. But this suggestion is not supported by the clinical observations of Dakin, of Foster, and of Beddard and myself; and the rapid *post mortem* degeneration which diabetic tissues often undergo might well account for the failure to find prosecretin *after death* in certain cases.

I wish to express my indebtedness to Dr. Beddard, Dr. Andrewes, and Dr. Langdon Brown for their kindness in supplying me with *post mortem* materials and with notes of the cases.

The expenses of this investigation were defrayed by a grant from the Royal Society.

References

(1) Bainbridge and Beddard, *this Journal*, Vol. I, p. 429, 1906.
(2) Bayliss and Starling, *Proc. Roy. Soc.*, Vol. LXIX, p. 352, 1902.
(3) Dakin, *Journ. Biol. Chemistry*, Vol. II, p. 305, 1907.
(4) Foster, *Ibid*, p. 297.
(5) Moore, Edie and Abram, *this Journal*, Vol. I, p. 28, 1906.

THE PRESENCE OF A NITRATE REDUCING ENZYME IN GREEN PLANTS

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Introduction

The question as to the form in which nitrogen is most easily assimilated by the green plant has long been under debate, and very conflicting statements have been made by various investigators :—

Thus Boussingault's[1] observations showed that nitrates seemed the most suitable form for the absorption of nitrogen by the plant.

Treboux[2] found that—

1. Nitrites are probably useful to the plant in alkaline solution, but poisonous in acid solution.
2. Nitrates have the same, if not a greater value than nitrites.
3. Ammonium salts are still better than nitrates or nitrites.
4. Amino acids and amides can be used but their nutritive value is much less.

He suggests that amino acids are decomposed by enzymes with liberation of ammonia.

Mazé[3] thinks that nitrates and ammonium salts are of equal value in metabolism.

1. Boussingault, *Agron.*, Tome I, pp. 69, 130, 1860.
2. Treboux, *Chem. Centr.*, p. 1619, 1905, from *Ber. deut. bot. Ges.* XXII, p. 570-572.
3. Mazé, *Compt. rend.*, 1899, pp. 128, 185-187.

Godlewski[1] found that higher plants when kept in darkness could produce proteids from nitrates and from the decomposition products of proteids, but in the case of the higher plants the assimilation of these substances is restricted in the absence of light. The necessary energy for nitrate assimilation is supplied by metabolism and respiration.

Laurent, Marchal and Carpiaux[2] found that plants kept in distilled water containing ammonium sulphate and saccharose respectively were able to assimilate these substances when placed in the light.

Hansteen[3] showed that nitrates were assimilated to a small extent in darkness.

Laurent[4] maintains that neither ammonium salts nor nitrates are assimilated in the dark.

Zuleski's[5] results show the possibility of proteid formation in the dark.

Suzuki[6] found that if plants were fed on 1 to 10 per cent. sugar solution, assimilation of nitrate and subsequent formation of proteid took place in the dark, as well as in the light. Plants containing a large amount of sugar were able even in the dark to form proteids from nitrates without the addition of sugar solution.

The observations of Suzuki would seem to suggest that in the case of Godlewski's experiments, and in those of Laurent, the plants had not contained any reserve carbohydrate, and were, therefore, dependent for their sugar upon the supplies formed during their exposure to the light.

The same explanation might hold for the other conflicting statements upon this point, as for example, those of Hansteen. From our point of view it is interesting to note that there are statements

1. Godlewski, *Bul. Acad. Cracow*, Vol. VI, p. 313, 1903.

2. Laurent, Marchal and Carpiaux, *Bied. Centr.*, Vol. XXVII, 1898, pp. 821-823; from *Bul. Acad. Belg.*, Vol. XXXI, pp. 815-865, 1896, and *Bot. Centr.*, Vol. LXX, p. 232, 1897.

3. Hansteen, *Ber. deut. bot. Ges.*, Vol. XIV, p. 368, 1896.

4. Laurent, *Bul. Acad. rag. Belg.*, *J. C. S. Abstracts*, Vol. II, p. 323.

5. Zuleski, *Ber. deut. bot. Ges.*, Vol. XV, p. 336, 1897.

6. Suzuki, *Bul. Coll. Agr.*, *Imp. Univ.*, Tokyo, Vol. III, pp. 488-507, 1898.

by various investigators pointing to nitrates as the best source of nitrogenous food for the green plant, and there are indications to show that this may be correlated with the presence of carbohydrates formed in photo-synthesis.

As the nitrogen is usually regarded as present in the proteid molecule chiefly in the form of NH_2 groups, there must obviously be a very efficient reducing apparatus in the green plant capable of converting the received grouping NO_3 into the necessary NH_2 form.

There are very few statements in the literature of plant physiology suggesting that such is the case, but one or two cases of the existence of a nitrate reducing enzyme have been recorded :—

Abelous and Aloy[1] showed that an enzyme capable of reducing nitrates to nitrites and nitrobenzene to aniline, is found in animal structures. They also demonstrated the presence of a similar enzyme in potato tubers.

Kastle and Elvolve[2] confirmed its presence in the potato, and showed that it was also present in the fruit of the egg plant (*Solanum melongina*).

Weehuizen[3] found that nitrous acid was present in the leaves of *Erythrina*, and concluded it was set free from a glucoside by the action of an enzyme; because if the leaf were killed by immersion in boiling water for thirty seconds, no nitrous acid was formed.

It seems very probable that Weehuizen's enzyme was the nitrate reducing enzyme which has formed the subject of this paper. There are also records of nitrate reducing bacteria.

Thus Burri and Stutzer[4] found that certain bacteria decomposed nitrate with liberation of free nitrogen, and that the action was increased in absence of air.

Also very recently Mattio Spica[5] has found that under anaerobic conditions yeast was able to reduce nitrates.

1. Abelous and Aloy, *Compt. rend. Soc. Biol.*, Vol. LV, p. 1080, 1903.
2. Kastle and Elvolve, *American Chemical Journal*, Vol. XXXI, pp. 606-641, 1904.
3. Weehuizen, *Pharm. Weekblad*, Vol. XLIV, pp. 1229-1232, 1907.
4. Burri and Stutzer, *Ann. Agron.*, Vol. XXII, pp. 491-494, 1896, from *Centr. Bact.*, Par. 1895, Vol. I, p. 2, Abt. 257, 350., pp. 392-422.
5. Mattio Spica, *J. C. S. Abstracts*, October, 1907.

It is clear that a more general distribution of such an enzyme is to be expected if nitrates are utilised in the formation of proteids. The present paper is the outcome of work carried out upon this hypothesis.

Experimental

Water plants (*e.g.*, *Elodea*, *Vallisneria*, etc.) were used in the first experiments on account of the greater facilities which these plants offered for collecting and examining any gases which might be liberated.

The following experiments were set up :—

A.—*Elodea* was placed in boiled tap water containing 0·5 gramme of asparagin and 1 gramme of potassium nitrate per litre. The plant was placed under an inverted funnel in the solution, and a test tube filled with water was put over the stem of the funnel to collect any gases which might be given off during the experiment. Thymol was added for antiseptic purposes to E and F. This was left in the light for two days.

B, C, D, E and F were set up in the same way.

B.—*Elodea* in a solution containing asparagin and potassium nitrate in the same proportion as in A but placed in the dark.

C.—*Elodea* boiled for some time, before putting it into a solution containing asparagin and potassium nitrate, and then placed in the light.

D.—Boiled *Elodea* put into a solution of asparagin and potassium nitrate and then placed in the dark.

E.—Chloroformed *Elodea* in a solution of asparagin and KNO_3 in the dark.

In this case the protoplasm would be killed, but many of the operative ferments present in the plant might remain.

F.—Chloroformed *Elodea* in a solution of asparagin and potassium nitrate placed in the light.

No gas was evolved on the first day, but on the second it was found that gas had been given off from the *Elodea* in A, B, E and F, but not in C and D. The gas was collected in a Winkler Hempel apparatus and analysed.

A.—Total volume = 24·6 c.c.

1. Passed through KOH, bulbs = 24·6 c.c.
2. Treated with KOH and pyrogallol, vol. = 24·6.
3. Sparked with oxygen and treated again with pyrogallol to absorb the oxygen = 24·6.

$\therefore$ Gas = nitrogen.

B.—Total volume = 25·6 c.c. This, treated in a similar manner, also proved to consist solely of nitrogen.

E.—Total volume = 37·5 c.c., which proved on analysis to consist of 37·5 c.c. nitrogen, 1·2 c.c. carbon dioxide.

F.—Total volume = 19 c.c. On analysis, only nitrogen present.

All analyses were carried out as described for A. These experiments were carefully repeated, and in all cases nitrogen was found to be given out by normal and chloroformed plants, but not by the boiled ones.

The solutions of asparagin in which the *Elodea* had been placed were examined: A, B, E and F were found to contain nitrites by the starch and potassium iodide test. Before the experiment they contained only nitrates. Therefore, during the experiment the nitrates were reduced to nitrites.

Substitution of Ammonium Salts for Nitrates.—Experiments were set up with an equivalent amount of ammonium sulphate in the place of nitrate, but no gas was given off, either in the light or in the dark, when *Elodea* and asparagin were placed in the solution.

The following Explanation is suggested for the Evolution of the Nitrogen:—

The nitrite formed by the reduction of the nitrate is converted into nitrous acid by the slightly acid cell sap. This in turn acts upon the asparagin giving malic acid and nitrogen.

(i) $2KNO_3 \rightarrow 2KNO_2 + O_2$

Owing to the nature of the reducing action the oxygen is not liberated as a gas, but retained in some form.

(ii) $KNO_2 \rightarrow HNO_2$

(iii) $$2HNO_2 + \begin{array}{l} CH \cdot NH_2 \cdot COOH \\ | \\ CH_2 \cdot CONH_2 \end{array} \rightarrow \begin{array}{l} CHOH \cdot COOH \\ | \\ CH_2 \cdot COOH \end{array} + 2N_2 + 2H_2O$$

The reaction came to an end after a few days in the dark, and the *Elodea* was then found to contain no starch.

The necessary acid medium for the reaction is probably provided by the cell sap. The oxidation of the carbohydrates may provide the energy necessary for the reaction, and its cessation may be due to the exhaustion of the supply of carbohydrates in the plant.

This is suggested by the fact that the reaction is accelerated, or restarted when it has stopped, by the addition of cane sugar, or glucose, to the solution.

Support is given to this theory by the fact that very little sugar, if any, was present in the plant which had been kept in the dark, whereas in the ordinary plant there was an appreciable supply of carbohydrates. An estimation of total sugars and starches in normal *Elodea* gave as a result 1·268 per cent. of dry weight as sugars and starch, while some of the same crop of *Elodea*, after placing in a solution of nitrate and asparagin in the dark, until no gas was liberated, yielded upon estimation merely a trace of carbohydrates.

From these experiments it thus seems probable that malic acid is formed in the plant in proportion to the nitrogen evolved as gas. It ought, therefore, to be possible to detect the malic acid in the plant itself.

Elodea was treated as described for A in the previous series of experiments. The plant was then finely ground, and shaken with a little water for two hours, and left overnight to extract. The solution was then filtered and treated with lead nitrate. A precipitate, presumably lead malate, was thrown down. The solution and precipitate were then heated, when the precipitate partially re-dissolved. The solution was then boiled to coagulate the proteids, and afterwards filtered, a clear solution being thus obtained. On concentrating and cooling small colourless crystals separated out. These were examined microscopically and found to have a similar structure to those of lead malate. They were re-dissolved in water, treated with a solution of calcium nitrate, which brought down a precipitate of calcium malate. This was filtered off, and the filtrate concentrated. No crystals came out, so it was concluded that all the malic acid had been removed as calcium malate.

This process involves the formation of nitrate as an intermediate product in metabolism, and nitrite is supposed to be poisonous to the plant. Experiment seems to suggest that in dilute solutions the protoplasm is not killed, and in stronger solutions ferment action is not arrested by the presence of nitrite.

Sprigs of *Elodea* were placed in solutions of nitrites of different strengths ranging from ·001 per cent. to 10 per cent. It was found that an enzyme present in all of them catalysed hydrogen peroxide, even after an immersion of three days in the nitrite solution. Trials made to plasmolyse the leaf cells after this immersion in solutions of potassium nitrite, indicated that in all the solutions used that were above 0·1 per cent. in concentration, the protoplasm was killed even after twenty-four hours.

But the amount of nitrite formed by the plant under normal conditions would probably be very minute at any moment, and would be removed almost immediately.

Extraction of an Enzyme capable of Reducing Nitrate to Nitrite.—Grass was dried at the temperature of the air, powdered, treated with water and left overnight to extract at 30° C. Chloroform was added for antiseptic purposes.

The solution so obtained was filtered, and treated with alcohol to precipitate the enzyme. This was filtered off, washed and dried.

The following experiments were then set up :—

1.—Enzyme in a solution of glucose, asparagin and potassium nitrate in water.

2.—Enzyme in a solution of cane sugar, asparagin and potassium nitrate.

3 and 4.—Controls to 1 and 2 in which the enzyme had been boiled for some time.

5.—Enzyme in a solution of asparagin and glucose but with no potassium nitrate present.

6.—Control to 5 ; containing the enzyme after prolonged boiling.

After twenty-four hours gas was being liberated by 1 and 2, but not by 3, 4, 5 or 6.

The glucose and cane sugar experiments had approximately the same volume of gas in each, but only about 1·5 c.c. was obtained in

either case. No analysis was made, but from its small solubility it was concluded that it was not carbon dioxide. The enzyme obtained in a similar way from *Elodea* gave the same results. At a later date this experiment was repeated upon a larger scale, and after two unsuccessful attempts, sufficient gas was obtained to make quantitative analysis quite possible.

By extraction from a very large bulk of the dried plants 0·5 gramme of the dried and powdered enzyme was obtained, though, of course, still in a very impure state.

This was placed in water containing respectively 2 per cent. of potassium nitrate, asparagin and dextrose.

The enzyme, which only partially re-dissolved after drying, was placed in a test tube containing the solution ; over this was inverted a slightly larger test-tube, and both these were placed in a larger dish of the solution.

In this way owing to the slowness of the outward diffusion of the enzyme, very little gas was lost as a result of its liberation taking place outside the walls of the larger test-tube.

The reaction proceeded in an incubator kept at 30° C. from November 28th until December 11th ; thymol was used as an antiseptic, and on the later date when the experiment ceased, there was not the slightest indication of bacterial activity in the solution.

During the first week the liberation of gas was very slow, but latterly it collected more rapidly, and at the end of the experiment 6·2 c.c. were available for analysis.

This gas underwent no change in volume, either over strong caustic potash or upon treatment with pyrogallol and potash (solutions made up according to Clowes'[1] formula) ; it was therefore concluded that the only gas present was nitrogen.

A nitrate reducing enzyme has also been found to be present in the following plants :—*Potamogeton*, *Vallisneria*, *Iris*, *Vicia faba*, various Gramineae.

1. Clowes, *Brit. Association Report*, 1896, p. 74.

In the case of *Vicia faba*, it was found in all parts of the plant, in root, stem, and leaves; but the reaction was longer in starting, and slower in progress in the case of the roots when placed in the nitrate and asparagin solution. As far as our experiments go, there seems no reason to doubt its very general distribution in plants.

Conclusions

The presence of a reducing ferment in green plants seems to have been established by means of this reaction with asparagin. It is not intended to suggest that this actual reaction occurs normally to any great extent in green plants, as the asparagin occurring in such plants is presumably to be regarded as an upgrade stage in the synthesis of proteids.

Further, as asparagin occurs to a considerable extent in such plants, it seems essential that the centres of nitrate reduction and of proteid formation must be quite distinct.

The reaction is to be regarded as abnormally wasteful in the plant economy, and not occurring in nature to any appreciable extent. Its occurrence under the experimental conditions has to be regarded as being due to the excess of both nitrate and asparagin in the solutions in which the plants were placed.

Possibly, under the conditions existing in ensilage, and in similar cases, the loss of nitrogen that takes place in the slowly decomposing heaps of grass may be due in part to the evolution of gaseous nitrogen, owing to the distribution of the enzyme becoming, as it naturally would, less localised.[1]

In the normal plant the only conditions necessary for nitrate reduction seem to be the presence of the enzyme, found in roots, stems, and leaves, and a suitable carbohydrate. The latter condition suggests the green leaf as the centre of reduction, and this agrees with the distribution of nitrates in the plant.

Our results seem to show that any hexose or polysaccharide is suitable for the supply of energy for nitrate reduction ; not as in

1. Evolution of gaseous ammonia takes place at the same time, and probably accounts to a large extent for the loss of nitrogen that occurs.

later stages of proteid synthesis where, according to Borodin[1] and Hansteen,[2] glucose is the only carbohydrate which, together with asparagin, can provide the necessary basis for construction of these bodies.

In conclusion we wish to thank Mr. J. H. Priestley for his valuable advice and helpful criticism throughout the progress of the work, and writing of the paper.

Our thanks are also due to Dr. F. F. Blackman and Mr. F. L. Usher for kindly criticism and suggestions.

1. Borodin, *J. C. S.*, *Abstracts*, Vol. II, p. 323, 1899.
2. Hansteen, *Chem. Centr.*, Vol. I, p. 295, 1897, from *Ber. deut. Ges*, Vol. XIV, p. 362-371

OBSERVATIONS ON THE SIGNIFICANCE OF THE HAEMOSOZIC VALUE OF THE BLOOD SERUM

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The term haemosozic value of the serum has been made use of to indicate those constituents present in the serum which preserve the red blood corpuscles from solution.[2] It is well known that on a gradual dilution of the plasma of the blood with distilled water we arrive at a point when the red blood corpuscles are broken up and the haemoglobin goes into solution—the blood is then spoken of as 'laked blood.' Based on this reaction Wright and Kilner[3] and later Wright and Ross,[4] described a new method of testing the blood and urine, and shewed that by means of the relationship existing between the haemosozic value of the serum and the haemosozic value of the urine we had a means of discriminating between physiological albuminuria and the albuminuria of renal disease.

Working on the lines laid down by the above-mentioned authors, I found[5] that the excretory quotient test, *i.e.*—

$\dfrac{\text{the haemosozic value of the urine}}{\text{the haemosozic value of serum}}$ gave values in the different forms of anaemia, and particularly in the anaemia of ankylostomiasis, very similar to those found to be the case in organic disease of the kidneys; viz., the value is always below that observed in health.

1. Dated Calcutta, December 5th, 1907.
2. Armand Ruffer, *British Medical Journal*, 1903, 1904.
3. Wright and Kilner, *Lancet*, April, 1904.
4. Wright and Ross, *Lancet*, October, 1905.
5. McCay, *Lancet*, June, 1907.

Reference to the literature of the subject will show that in healthy Europeans the excretory quotient is always represented by the figure 2 or over, and in Bengalis by 1 or over; while in nephritis or anaemia with oedema, as the case may be, it falls much below 2 in Europeans, and usually well under 1 in Bengalis.

A full description of the technique of the method of carrying out the test will be found in the numbers of the *Lancet* referred to, and need not detain us.

The points with which the present paper will deal are :—

(i) *On what does haemolysis caused by dilution really depend.*

(ii) *What is measured by an estimation of the haemosozic value—*

(1) *In health,*

(2) *In disease.*

(iii) *The significance of the modification of the haemosozic value of the serum by drugs and the bearing of a lowering on the incidence of blackwater fever.*

I.—The Cause of the Solution of the Red Blood Corpuscles on Dilution of the Plasma

The blood may be looked upon, so far as the erythrocytes and plasma are concerned, as a mass of impermeable or slightly permeable globules floating in a fluid medium of the same density. Very careful measurements by Krönig and v. Fürth[1] have shewn that the freezing-point of the blood-plasma, or of the blood serum and blood-corpuscle pulp is the same ; and the same osmotic equilibrium has been shewn to exist between the various body fluids, with the exception of the urine.

It may, therefore, be accepted that the osmotic tension of the plasma and of the corpuscles is identical ; any modification of the density of the plasma, as, for instance, its dilution by distilled water, will upset the osmotic equilibrium existing between the corpuscles and the plasma, and by osmosis water will tend to pass into the red blood corpuscles and the salts to pass out.

1. *Monatschr. für Geburtsh. u. Gynäk.*, 1901.

In practice it has been found that a dilution of the plasma by a weaker and weaker saline solution (two volumes of the diluted saline being added to one volume of blood) gives a very definite point when haemolysis takes place—the haemoglobin going into solution in the plasma. This point may be termed the haemolytic point.

To make the reaction clear it might be expressed in the form of an equation :—

If x equals the total salt concentration of the plasma of the blood examined, and it is found that—

$$1 \text{ vol. of blood} + 2 \text{ vols. of a } \frac{N}{45} \text{ NaCl} = \text{haemolysis,}$$

$$\text{normally } x = \text{about } 0{\cdot}85 \text{ per cent.,}$$

$$\text{and } \frac{N}{45} = 0{\cdot}130 \text{ per cent.,}$$

substituting these values in the equation—

$$= 1 \text{ vol. of } 0{\cdot}85 \text{ per cent. solution} + 2 \text{ vols. of } 0{\cdot}130 \text{ per cent. solution,}$$

$$= \frac{0{\cdot}85 \text{ per cent.} + 2\,(0{\cdot}130 \text{ per cent.})}{3} = 0{\cdot}370 \text{ per cent.}^{1}$$

that is, the salt concentration of the plasma in the example at which haemolysis takes place is a concentration of 0·370 per cent.[1]; in other words, the plasma of the blood will bear dilution until it becomes reduced from a 0·85 per cent. solution to a 0·37 per cent. solution before any general breaking up of the red blood corpuscles takes place. The fact that haemolysis does take place on a dilution of the plasma shows that the envelope of the red blood cells is impermeable to contained salts and haemoglobin ; if this were not the case, these would wander out until the osmotic tension of the plasma and inside the corpuscles reached equilibrium and no haemolysis would occur. Instead of this, the salts and haemoglobin of the corpuscles not being able to pass out, water finds its way in by endosmosis, the corpuscles swell up and, at the degree of dilution corresponding to the haemolytic point, sudden and complete disruption of the erythrocytes takes place and the blood is laked.

1. I have purposely taken the haemolytic point very low. In the blood of man haemoglobin is extruded from the red cells at a concentration of a sodium chloride solution of 0·47 per cent.

We have, therefore, in this dilution method a very definite means by which the degree of dilution of the plasma or serum necessary to cause haemolysis can be estimated, and by using diluted normal saline solutions, as introduced by Wright, its value can be expressed in terms of NaCl.

So far as our theoretical knowledge of the physical chemistry of the blood goes, it would appear that the solution of the red blood corpuscles brought about by a dilution of the plasma with distilled water or a weak saline solution, as the case may be, depends on a difference of osmotic tension inside and outside the red blood corpuscles —haemolysis taking place when the osmotic pressure within the corpuscles is able to overcome the resisting power of the corpuscles to disruption.

II.—What is Measured in an Estimation of the Haemosozic Value of the Serum

1. *In health.*—As indicated above the haemosozic value of the plasma or serum should vary distinctly with its osmotic pressure, which in its turn depends on the total number of molecules in solution.

Neglecting the large proteid molecules, which have very little effect on the osmotic pressure, it would appear that the haemosozic value is really a measure of the total salt concentration or total salinity of the plasma or serum, *i.e.*, the total number of inorganic molecules in solution.

The evidence that this is the case :—

(i) The specific electrical conductivity of fluids tested by Dr. Waller was found to be roughly proportional to the salt content as estimated by this dilution method.[1]

(ii) Working mainly on Bengalis over a series of eighty-four observations, I have found[2] the salt content as estimated by the dilution method to be 1·054 per cent. expressed in terms of NaCl. This is a slightly higher figure than that given for the percentage of salts in the serum in Europeans but, by actual quantitative analyses, I found

1. *Lancet*, quoted in Wright and Kilner's paper.

2. *Scientific Memoirs;* 'Metabolism of Bengalis'; in press.

that in healthy Bengalis the average percentage of total salts present in the blood was 1·06 per cent.—a figure practically identical with the average salt concentration obtained by the dilution method.

While it may be admitted that the electrical conductivity is a more accurate measure of the number of inorganic molecules in the serum than the amount of ash it yields, still quantitative estimations give a fairly close approximation to the total salts present.

It may, therefore, be accepted that for all practical purposes in estimating the haemosozic value of the serum in healthy individuals, what is actually measured is the total salt concentration or total salinity.

2. *In disease.*—In the observations recorded by Wright and Kilner, and by myself, on the salt concentration of the serum in anaemic conditions, it will be seen that the serum is modified in the same characteristic manner as found in estimations of the haemosozic value in nephritis with oedema—the serum giving in these cases a very high figure for its haemosozic value.

Some examples of the results are shewn in Table I.

TABLE I

Reference	Authority	Disease	Haemosozic value of serum in terms of NaCl	Remarks
Lancet, 1904	Wright and Kilner	Pernicious anaemia	2·4%	1,700,000 Red blood corpuscles
Do.	do.	Chlorosis	1·56%	
Do.	do.	do.	3·00%	Oedema of legs
Do.	do.	Anaemia	2·40%	Gastric ulcer
Do.	do.	do.	2·44%	Thrombosis of veins of legs
Lancet, 1907	McCay	Anaemia	2·12%	Ankylostomiasis; oedema and ascites
Do.	do.	do.	6·09%	Do. Death
Do.	do.	do.	3·95%	Oedema and ascites
Do.	do.	do.	2·32%	Ankylostomiasis, marked oedema
Do.	do.	do.	1·72%	

From these results and from other evidence brought forward it appeared probable that in anaemia and in oedema from whatever cause there was an increase in the total salts of the blood, and particularly an actual retention of chlorides by the tissues and blood plasma.

While not denying that in oedema and nephritis there is retention of chlorides so that the system and blood contains an *absolutely* larger amount of total salts, it appeared most improbable that the blood plasma should possess such a *relatively* high percentage as that obtained for the haemosozic value in Table I.

In order to clear up the mystery of these high percentages of salt of the serum—in one instance as high as 6·0 per cent.—a further investigation was undertaken, several different lines of research being resorted to. As the results obtained would appear to be of some importance, I make no excuse for giving them in detail.

A.—Investigations to Exclude the Possibility of a Combination of Salts with Colloid Material

It seemed absurd to suppose that in the series of cases shewn in Table I there could be anything like the large percentage of *free* salt present in the serum which the estimation of the haemosozic value indicated ; further, it appeared probable that some of the salt might be combined with the colloids of the serum and that, on manifold dilution with distilled water—as actually takes place in the estimation of the haemosozic value—the combined salt gradually becomes dissociated, and coming off *pari passu* with the degree of dilution, maintains for some time the isotonicity of the diluted serum and the red blood corpuscles. Eventually, however, as the serum becomes more and more diluted its salt concentration falls below the point necessary for the maintenance of the integrity of the red blood corpuscles, and haemolysis takes place.

To put this view to the test a number of cases, in which the haemosozic value was high, were examined with regard to the percentage of chlorides in the serum before and after a twenty-fold dilution with distilled water. The results obtained are shewn in Table II.

TABLE II

Case	Date	Haemosozic value of serum in terms of NaCl	Percentage of chlorides of serum before dilution	Percentage of chlorides of serum after dilution
1	14/7/07	1·64%	1·05%	1·06 %
2	17/7/07	3·24%	0·70%	0·75%
3	29/7/07	1·358%	0·724%	0·73%
4	28/8/07	5·44%	0·692%	0·692%
5	14/9/07	3·18%	0·668%	0·688%
6	19/11/07	3·46%	0·719%	0·734%
7	21/11/07	2·26%	0·485%[1]	0·498%
8	22/11/07	0·97%	0·691%	0·691%

The table shews no definitely large increase in the percentage of chlorides of the serum either before or after a twenty-fold dilution with distilled water. We may, therefore, safely conclude that whatever is the cause of the high percentage of the haemosozic value of the serum in anaemia and oedema it cannot be due to a retention of the chlorides in combination with a colloid as Förster and Marie are inclined to believe.

The percentage of NaCl in the serum is, of course, no guide in determining to what extent retention of chlorides has taken place, for the hydraemic condition of the serum may conceal a great increase in NaCl and, further, the tissues may take up a large quantity of NaCl.

This is probably the case in oedema.

B.—INVESTIGATIONS UNDERTAKEN WITH THE VIEW OF DETERMINING WHEREIN LAY THE ACTUAL CAUSE OF HIGH HAEMOSOZIC VALUE

So far in these investigations I had firmly believed that the high haemosozic values were due to something—most probably inorganic salts—present in the serum in much greater proportion than in health and, therefore, requiring a larger number of dilutions to lower the osmotic pressure of the serum sufficiently to cause disruption of

1. Oedema disappearing may explain the low percentage of chlorides.

the red blood corpuscles. Having failed to find any marked increase in the chlorides of the serum, I began to suspect some change in the resisting power of the erythrocytes as a likely explanation.

In order to test this view, the following method was devised nd carried out in a series of cases presenting a high haemosozic value :—

The haemosozic value of the patient's serum was estimated in the usual way—his own red blood corpuscles serving as the indicator of haemolysis. Having obtained the figure representing the haemosozic value of the serum in terms of NaCl, a sample of the same serum was now tested for its haemosozic value, not against his own red blood corpuscles, but using the red blood corpuscles of a normal individual as the indicator of haemolysis. The results obtained in this way are very striking and throw considerable light on the question at issue ; they are shewn in Table III.

TABLE III

Case	Date	Haemosozic value of serum *versus* own red blood corpuscles	Haemosozic value of serum *versus* normal red blood corpuscles	Remarks
1	17/7/07	3·24%	0·984%	Anaemia and oedema
2	29/7/07	1·358%	0·89%	Bright's disease, etc.
3	30/7/07	1·69%	1·06%	Do.
4	16/8/07	5·44%	1·06%	Ankylostomiasis anaemia and oedema
5	19/11/07	3·46%	1·02%	Do.

It is evident from the results obtained in this way that in using the patient's own red blood corpuscles as an indicator a far larger number of dilutions with distilled water of his serum are necessary to effect haemolysis than when the red blood corpuscles from a healthy individual are acting as the indicator. In both cases it is the serum from the same patient that is diluted, the indicator—red blood cells—alone changing, yet it has been found that the serum may require up to a thirty- or forty- fold dilution to effect haemolysis of his own erythrocytes, whereas a six- or eight- fold dilution will be sufficient to cause disruption of normal red blood cells. These

figures would, therefore, appear to point to a greatly increased resisting power of the erythrocytes in those conditions in which the haemosozic value of the serum is high, and not to a change in the total salinity of the serum whereby its osmotic pressure is greatly enhanced. We may, therefore, conclude that the increased resisting power of the red blood corpuscles to haemolysis in the several conditions examined does not depend on an increased salt concentration of the plasma—as the results obtained in healthy individuals would lead us to believe—and it cannot be explained by physical reasons alone.

Some other factor is present which in some way is able to increase the resisting power of the red blood cells to disruption. What that factor is, it is impossible to say at present. It may be that in some way the permeability of the erythrocytes is greatly increased—as for instance by the presence of ammonium salts—thus allowing of a free exchange of inorganic matter between the corpuscles and the diluted plasma, or, what would appear to be more likely, a new substance is present which is able to prevent a breaking down of the red blood corpuscles in the diseased conditions in which a high haemosozic value of the serum is found. The evidence that something capable of doing this does exist will be discussed below.

C.—Investigations Undertaken to Shew that in certain Diseased Conditions the High Haemosozic Value is no Criterion of the Actual Quantity of Salts Present in the Blood

Having failed to find evidence of any great increase in the chlorides or of the salt concentration when measured against normal red blood cells, I next turned to actual estimations—quantitatively—of the percentage of salts in the blood of individuals shewing a high haemosozic value.

It will be fairly obvious that, if the blood in the particular conditions shewing such high values for the haemosozic power does really contain a higher—much higher—percentage of salts; and if, due to this, the haemosozic value is greatly increased, then by quantitative analysis this increased salinity should be demonstrated.

The following table—Table IV—gives the facts obtained by analysis :—

TABLE IV

Case	Date	Disease	Haemosozic value of serum in terms of NaCl	Total salts of blood	Total solids of the blood
1	28/8/07	Marked anaemia and oedema general	5·444%	1·20%	10·579%
2	14/9/07	Anaemia and oedema	3·18%	1·103%	16·176%
3	19/11/07	Do.	3·46%	0·987%	12·862%
4	21/11/07	Do.	2·26%	0·988%	15·563%
5	Do.	Heart oedema	0·848%	0·926%	14·646%

These results would appear to be absolute proof that while the haemosozic value of the serum in health corresponds very closely with its total salts, in certain diseased conditions characterised by a high haemosozic value—notably nephritis, anaemia and oedema generally—the haemosozic value is no measure whatsoever and in no way corresponds to the actual percentage of salts in the blood or serum. Again, by this method of investigation the conclusion is forced on us that some unknown factor, other than an increase in the percentage of salts in the blood, is present able to increase the resisting power of the red blood corpuscles.

Further, from the investigations carried out in cases of marked oedema it would appear that there is very little increase in the percentage of salt present—the blood being able in some way or other to maintain its chemical composition at a fairly uniform level, at least so far as its inorganic salts are concerned.

III.—The Significance of the Modification of the Haemosozic Value of the Serum by Drugs, and the Bearing of a Lowering on the Incidence of Blackwater Fever

As it was evident from the results recorded above that the haemosozic value of the serum or, more accurately, perhaps, the resisting power of the red blood corpuscles varied within very wide limits in disease, it appeared to be a matter of some importance to determine the action of certain commonly used drugs, and more particularly what effect 'quinine' had on the resistance of the erythrocytes.

In blackwater fever there is undoubtedly a great and wide-spread breaking down of the red cells of the blood ; it was, therefore, evident that any drug, the absorption of which caused a lowering of the resisting power of the red cell, would, other things being equal, tend to bring those cells within the danger zone and precipitate an attack of blackwater fever.

In blackwater fever it is generally accepted that there is a virulent malarial infection primarily—the infection probably being repeatedly effected daily—so that many of the red cells have been already broken up, many others on the point of breaking up, and a large number of those remaining injured by the malarial parasite ; if now, for any reason the salt concentration of the plasma becomes seriously lowered the effect will be to bring the red corpuscles—and particularly the innumerable injured corpuscles—nearer and nearer to their haemolytic point according to the greater and greater lowering of the total salinity of the plasma. That it is those corpuscles, injured by the presence of the malarial parasite, that do break up in blackwater fever is evident from the fact that no parasites can be found, once the attack is precipitated, the corpuscles hitherto containing them having disappeared on disruption.

In order to obtain information of the effects of malaria and its treatment by 'quinine' on the salt concentration of the blood, a series of observations was begun. However, as malaria during the period was rare in Calcutta, I was forced to carry out most of the investigation on healthy individuals, but the results in the few cases of malaria treated with quinine sulphate shew exactly the same modification of the plasma as met with in health on administration of the drug.

The following table makes clear this modification due to the particular drugs administered. In order to check the results, a chemical estimation of the chlorides of the blood serum was made before and after the drug was given, and, as will be seen, the results are in harmony with the changes observed in the salt concentration.

TABLE V

Case No.	Date	Disease	Haemosozic value of serum in terms of NaCl (before)	Drug and dose	Haemosozic value of serum in terms of NaCl (after)	Chlorides of serum before drug	Chlorides of serum after drug
1	20/7/07	Healthy	0·984%	Quinine Sulph. gr. 30	0·754%	—	—
2	23/7/07	Do.	0·85%	„ gr. 20	0·808%	—	—
2	25/7/07	Do.	0·85%	„ gr. 30	0·758%	—	—
2	26/7/07	Do.	0·85%	„ gr. 20	0·63%	—	—
2	27/7/07	Do.	0·85%	„ gr. 20	0·806%	—	—
3	29/7/07	Do.	0·888%	„ gr. 30	0·742%	—	—
3	31/7/07	Do.	0·888%	„ gr. 30	0·707%	—	—
3	1/8/07	Do.	0·888%	„ gr. 30	0·707%	—	—
4	3/8/07	Do.	0·965%	„ gr. 20	0·762%	—	—
4	5/8/07	Do.	0·965%	„ gr. 10	0·736%	—	—
5	7/8/07	Do.	1·07%	„ gr. 5	0·965%	0·753%	0·724%
5	9/8/07	Do.	1·07%	„ gr. 5	0·914%	0·753%	0·723%
5	10/8/07	Do.	1·07%	„ gr. 5	0·863%	0·753%	0·705%
5	12/8/07	Do.	1·07%	„ gr. 10	0·701%	0·753%	0·612%
5	13/8/07	Do.	1·07%	„ gr. 10	0·876%	0·753%	0·720%
5	14/8/07	Do.	1·07%	„ gr. 10	1·016%	0·753%	0·758%
5	15/8/07	Do.	1·07%	„ gr. 20	1·04%	0·753%	0·754%
6	23/9/07	Do.	0·928%	(Qui. Sulph. gr. XV, Mag. Sulph. gr. 30, Ac. Sulph. dil. Mi. 15)	0·779%	0·68%	0·676%
6	25/9/07	Do.	0·928%	Do.	0·861%	—	—
7	23/9/07	Do.	1·168%	Magnes. Sulph. gr. 30	0·928%	0·701%	0·684%
7	25/9/07	Do.	1·168%	Do.	0·812%	—	0·679%
7	26/9/07	Do.	1·168%	Do.	0·719%	—	0·675%
7	27/9/07	Do.	1·168%	Do.	0·719%	—	—
7	28/9/07	Do.	1·168%	Do.	1·04% (watery stools)		0·695%

The results shewn in above table are of very great interest; in every instance in which a sulphate was given by the mouth there was a well-marked fall in the haemosozic value of the serum measured in terms of NaCl by the dilution method. Further, it will be seen that the rapidity of the fall depended more or less on the strength of the dose.

Another important point, brought out in Nos. 2, 5, 6 and 7, is the recovery or rise to the normal of the haemosozic power when daily doses are administered on consecutive days. We might look on the early effects of sulphates on the haemosozic value as a negative phase which gradually passes off on regular administration of the drug.

The following table shews the results obtained in cases of malaria treated by quinine sulphate.

TABLE VI

No.	Date	Disease	Haemosozic value of serum in terms of NaCl *versus* normal red cells	Drug and dose	Haemosozic value of serum in terms of NaCl *versus* normal red cells	Remarks
1	22/7/07	Benign Tertian	0·928%	Quin. Sulph. gr. 20	0·776%	Blood examined on 4th day, *i.e.*, after Quin. Sulph. gr. 80 had been given
2	1/8/07	Malig. Tertian	1·004%	Quin. Sulph. gr. 30	0·97%	
	3/8/07	Do.	Do.	Do.	0·848%	—
3	24/8/07	Malig. Tertian	1·027%	Quin. Sulph. gr. 10	0·976%	10 grains given for two days
	29/8/07	Do.	Do.	Quin. Sulph. gr. 5	0·812%	5 grains for 5 days
4	26/8/07	Malig. Tertian	0·928%	Quin. Sulph. gr. 30	0·849%	—
5	26/8/07	Malig. Tertian	0·928%	Quin. Sulph. daily 45 grs.	0·742%	45 grains daily for two days

The same fall in the haemosozic value is seen as occurs in health when sulphates are administered. These cases were all examined by using the red blood corpuscles of a healthy individual as the indicator—the patients were in hospital, and a few drops of blood were drawn off and sent to the laboratory in exactly the same way as for Widal's test. It is important to note that by using normal red corpuscles as the indicator I eliminated any change in the resisting power of the patient's red cells due to causes other than changes in the total salt concentration of the blood.

The Significance of the Modification of the Haemosozic Value Effected by Sulphates as Shewn in Tables V and VI

In an earlier part of this paper I brought forward evidence to shew that the haemosozic value of the serum in health expressed in terms of NaCl gave results almost identical with those obtained by a quantitative estimation of the total salts of the blood.

In the few cases of malaria examined—using normal red blood corpuscles for an indicator—it may be accepted that the percentages obtained for the haemosozic value are an actual measure of the total salinity of the blood, both before and after the administration of sulphate of quinine.

Therefore, both in healthy individuals (Table V) and in malaria (Table VI) the lowering of the haemosozic value of the serum is really due to an actual diminution of the total salts of the blood, and not to some other cause such as a lessening of the resisting power of the red blood corpuscles. That this is the case we have a certain amount of corroborative evidence in the accompanying depression of the chlorides of the serum (*vide* Table V, Nos. 5, 6 and 7).

It is, therefore, evident that—other things being equal—this depression of the haemosozic value of the serum or, what would appear to be the same thing—so far, at least, as healthy individuals or those suffering from malaria are concerned—the lessened salt concentration of the blood, produced by the administration of sulphates, does upset the osmotic equilibrium existing between the red blood corpuscles and the plasma in which they float. The diminution in the number of inorganic molecules present in the plasma lessens its osmotic pressure, and the osmotic pressure inside the red blood cells remaining as before, the effect will be to tend to burst the corpuscles open. This effect will be the greater the more the inorganic molecules of the plasma are diminished in number; and disruption from this cause will, of course, be the more easily effected the more the corpuscle is injured by the presence of the malarial parasite.

As already stated, the haemolytic point of normal blood is about

0·37 per cent.,[1] *i.e.*, the red blood corpuscles will bear a dilution of the plasma until it becomes about equal to a 0·37 per cent.[1] solution before actual general haemolysis takes place. This fact can be easily verified by the simple experiment of mixing an equal volume of blood and distilled water together when, it will be seen, no general laking of the blood has occurred; but, on the further addition of a fraction of a volume of distilled water quite suddenly, on the proper dilution being attained, general haemolysis takes place. It might be argued from this that if the erythrocytes will bear a dilution of the plasma until its density is reduced from about a 0·85 per cent. solution to one of about 0·37 per cent.,[1] the lowering produced by sulphates—such as quinine sulphate, magnesium sulphate or acid sulph. dil.—would be quite insufficient to cause haemolysis. (The greatest reduction observed after the administration of sulphates in any of the persons examined only lowered the density of the plasma from a 1·07 per cent. solution to a 0·701 per cent. solution.) This is quite true so long as we are dealing with normal blood or even in conditions of ordinary malarial infection, otherwise haemolysis and blackwater fever would follow after the administration of sulphates in every case of malaria.

In order, therefore, to explain the onset of an attack of blackwater fever, it is necessary to bridge the interval between 0·63 per cent., the lowest salt concentration above recorded, and 0·37 per cent.,[1] the lowest depression of the density of the plasma *normal* corpuscles will bear.

A purely physical explanation might be advanced which would cover much of the ground. In blackwater fever we are dealing with a condition in which many of the red blood cells are invaded by the malarial parasite, resulting in injury to the cohesive power of the stroma and haemoglobin so that the resisting power of the cells is greatly lessened; it is, therefore, quite probable that a sudden lowering of the osmotic tension of the plasma due to a decrease in the number of inorganic molecules contained—brought about by the administration of sulphates—becomes the determining factor

1. Probably 0·47 per cent. NaCl is more correct.

in the breaking up of those injured cells. In other words, a difference of osmotic tension between the red cells and the plasma, which in health would have no haemolytic effect, in blackwater fever becomes a very important factor in the causation of the attack.

The question therefore arises :—*What other factor is there in blackwater fever the presence of which permits of haemolysis taking place under circumstances that would not occur in health or in ordinary malaria ?*

Two explanations are evident from the results above recorded. The first of these is the actual injury done to the red corpuscles by the presence of the malarial parasite ; that this in itself is not sufficient would appear probable from the fact that even severe malarial fever treated with heroic doses of quinine sulphate will not develop into blackwater fever, except in certain well-defined areas. The second explanation would rest on the probable formation within the system of an haemolysin or haemolytic ferment by the action of which the resisting power of the red cells becomes diminished so that, in extreme cases, solution takes place without any great change in the composition of the plasma ; but, in the majority of recorded cases, the power of resistance of the erythrocytes being diminished by the haemolysin, the lowering of the osmotic pressure of the plasma—due to the action of sulphates—becomes the determining factor, and sets up an attack. The presence of a substance such as this would completely explain the onset of haemolysis long before any such dilution of the plasma was attained as has been found necessary in health. The degree of dilution necessary for haemolysis would form a most interesting and important subject for research in patients just before the onset and during an attack of blackwater fever.

Once recovery has begun to set in, a condition such as found in ankylostomiasis would be expected, viz., a very greatly increased resisting power of the remaining red blood cells due, in all probability, to the formation within the system of an antihaemolysin.

That something in the nature of an haemolysin is present and is the cause of the great anaemia of ankylostomiasis seems now to be generally accepted. The anaemia is not due to a mere sucking of

blood by the ankylostoma—in fact, it is very doubtful if they suck blood at all.

The great increase in the haemosozoic value, which I have found to hold true in these conditions, and particularly when oedema is well marked, would appear from the evidence brought forward above, to depend on a greatly enhanced resisting power of the patient's red blood corpuscles. This high haemosozic value is made up of two factors : one, the ordinary haemosozic value, depending on the number of inorganic molecules present in the plasma ; the other, something most probably of the nature of an antihaemolysin, by virtue of which the erythrocytes of patients—suffering from ankylostomiasis for some time, or recovering therefrom—are endowed with a very great power of resistance compared with normal red corpuscles. A similar explanation must hold also for certain other forms of anaemia and for the anaemia of nephritis, as, even in renal disease, I have found a high haemosozic value is no measure of the actual total salts of the blood.

A body possessing haemolytic properties has been isolated from the tissues of the ankylostoma, and the formation of an antibody within the system would be expected ; indeed, the presence of such a substance forms the only reasonable explanation of the high resisting power of the erythrocytes in ankylostomiasis, at least in its later stages, the only stages I have had a chance of investigating.

General Summary of Causation of Onset of Blackwater Fever

Practically everyone agrees that the malarial parasite is the real cause underlying the condition, and that by prevention of malaria blackwater fever would become non-existent. The great majority of clinical observers consider 'quinine' the actual exciting cause precipitating an attack. This action of quinine is so widely believed in by those of great experience in the disease as to appear worthy of general acceptance. The word 'quinine' has been used very loosely in connection with malaria ; in probably 99 per cent. of instances of administration of the drug by the mouth, 'quinine'

means the sulphate. From the investigations and results recorded in an earlier part of this paper on the action of sulphates on the inorganic salts of the blood, it seems very probable that the lowering of the total salt concentration from the administration of sulphates, such as quinine sulphate, magnesium sulphate, etc., in a patient saturated with malaria, is quite sufficient to precipitate an attack. In all probability, besides the actual weakening of the erythrocytes from malarial infection, another factor, causing a lessened power of resistance of the red cells, comes into force, and there is reason to believe this is of the nature of a haemolytic toxin or haemolysin.

How this haemolysin is formed; the conditions necessary for its formation; why its formation is limited to malaria occurring within certain well-defined areas, etc., are questions which, for the present, cannot be answered. It is not improbable that the relative virulency of the parasites under different climatic conditions may be found to explain the mystery. For the present, it would appear, from the effects of sulphates on the salts of the blood, that the administration of quinine sulphate even in small doses may just make all the difference between a malarial fever of a special type and a malarial fever that developes into blackwater fever.

How the Haemosozic Value of the Blood may be Increased—a Probable Rational Indication for the Treatment and Prophylaxis of Blackwater Fever

As it was possible, by the administration of sulphates—probably also by giving potassium salts, alkaline carbonates and compounds of alkalis with vegetable acids—to decrease the total number of inorganic molecules in the blood plasma, and thus, in malaria, tend to produce haemolysis of the red blood corpuscles and perhaps precipitate an attack of blackwater fever, it seemed a rational indication to discover some means of increasing the total number of inorganic molecules of the plasma; perhaps, in that way, lessening the tendency of the red blood corpuscles to haemolysis and thus attain a position of being able to prevent the development of blackwater fever entirely.

The SO_4 of sulphates, probably on absorption, combines with the Na, Ca, etc., of the blood plasma, forming sulphates which are eliminated at once on arrival at the kidneys, thus decreasing the number of inorganic molecules in the plasma ; it, therefore, seemed possible by giving a salt that need not necessarily be eliminated at once—*i.e.*, one not absolutely foreign to the system—to be able to increase the total salts of the blood and thus raise the haemosozoic value of the plasma. The salts most likely to cause this increase were the chlorides. I therefore began a series of observations on the effects of the administration of chlorides by the mouth on normal individuals. As it was very necessary, in view of the malarial origin of blackwater fever, that quinine should be given, the form of chloride administrated was quinine hydrochloride.

The results obtained are shewn in Table VII.

TABLE VII

Case No.	Date	Haemosozic value of serum in terms of NaCl (before)	Drug given	Haemosozic value of serum in terms of NaCl (after)	Chlorides of serum before drug	Chlorides of serum after drug
1	17/7/07	0·903%	Quinine Hydrochloride gr. 25	1·044%	—	—
2	16/8/07	1·09%	Do. do. 20	1·09%	0·712%	0·723%
2	18/8/07	1·09%	Do. do. 20	1·09%	—	0·732%
2	19/8/07	1·09%	Do. do. 20	1·09%	—	0·719%
2	20/8/07	1·09%	Do. do. 20	1·170%	—	0·745%
2	21/8/07	1·09%	Nil.	0·899%	—	0·734%
3	21/8/07	0·865%	Quinine Hydrochloride gr. 10	1·04%	0·73%	—
3	23/8/07	0·865%	Do. do. 10	0·865%	—	0·719%
3	24/8/07	0·865%	Nil.	0·865%	—	—
3	25/8/07	0·865%	Nil.	0·959%	—	—
3	26/8/07	0·865%	Quinine Hydrochloride gr. XXX	1·392%	—	0·877%
3	27/8/07	0·865%	Do. do. gr. XXX	1·866%	—	0·740%
3	28/8/07	0·865%	Do. do. gr. XXX	1·856%	—	0·718%
4	21/11/07	0·865%	Quin. Hydrochlor. gr. XV, Sod. Chlor. gr. 90,, Ac. Hydrochlor. dil. Mi. XV	1·076%	0·723%	0·736%
4	23/11/07	0·865%	Do. do.	1·157%	—	—
4	24/11/07	0·865%	Do. do.	1·43%	—	0·964—
6	5/11/07	0·814%	Quin Hydrochlor. gr. X, Ac. Hydrochlor. Mi. X	1·016%	—	—

These results present a very different picture to that obtained from an administration of the sulphate of quinine or other sulphates. No lowering of the haemosozic value of the serum was observed in any one instance, and, in the majority of cases, the value was increased. That this increase is in part, at least, due to an increase in the number of inorganic molecules of the serum would appear probable from the accompanying increase in the chlorides of the serum. The increase is very marked when full doses of quinine hydrochloride are given, and this is further enhanced by an addition of sodium chloride and hydrochloric acid to the prescription.

The importance of the bearing of these results on the incidence of blackwater fever is obvious; the raising of the haemosozic value of the plasma in the treatment of malaria, especially in districts where blackwater fever is prevalent, should, other things being equal, be of great service in lessening the liability to its onset.

With the knowledge of the action of sulphates on the salinity of the blood gained from the above-recorded investigations, and what the significance of that action really is, it appears to me quite time that the treatment of malaria by quinine sulphate should cease, and that a fair trial should be given to its substitute, quinine hydrochloride or the acid hydrochloride. If this were done I think there is a reasonable hope that the number of cases of blackwater fever following almost immediately on the ingestion of sulphates in some form—and this covers the very large majority of the cases—would soon shew a rapid diminution, with a saving of many valuable lives yearly.

In connection with the views here put forward it would be interesting to obtain reliable information with regard to the relative liability to blackwater fever of those infected with the Ankylostoma Duodenale, and those—mostly Europeans—who are non-infected.

From the arguments made use of above I should expect that the high resisting power of the red blood corpuscles, characteristic of those suffering from Ankylostomiasis, would probably prevent altogether the occurrence of blackwater fever in such cases or, at least, lessen the likelihood of its onset.

Conclusions

1. The haemosozic value of the serum expressed in terms of NaCl corresponds with the total salt concentration.

2. In conditions of an increase in the haemosozic value above the normal, the haemosozic value is a measure of

   (1) The total salt concentration.

   (2) A factor other than the salt concentration increasing the resisting power of the red blood corpuscles; probably an antihaemolysin.

3. The lowering of the haemosozic power of the serum by the administration of certain drugs—sulphates, potassium salts, etc.—would appear to be a most important factor, under certain circumstances, in the precipitation of an attack of blackwater fever.

4. The raising of the haemosozic value of the serum by the administration of chlorides, such as quinine hydrochloride, sodium chloride, etc., would appear to be a rational indication for the prophylaxis and treatment of blackwater fever.

In conclusion, I wish to express my thanks to those who assisted me in the investigation by providing the material for examination. I am specially indebted to Dr. Upendra Nath Brahmachari, Campbell Medical School, Calcutta, for the great majority of the pathological cases investigated.

I desire also to thank Lieut.-Col. Drury, I.M.S., Major Chatterton, I.M.S., and Captain Mackelvie, I.M.S., physicians of the Medical College Hospital, Calcutta, for permission to examine the patients under their care.

Since writing the above paper I have had the opportunity, due to the kindness of Major L. Rogers, I.M.S., Professor of Pathology, Medical College, Calcutta—of putting the views advanced with regard to the causation of blackwater fever to some extent to the test.

The patient was recovering from a typical attack when the blood for examination was taken. The haemoglobinuria had disappeared, and the ordinary symptoms of the disease were rapidly subsiding.

At this period it was evident that the active cause of the haemolysis had disappeared and that in all probability the erythrocytes that had escaped disruption would be found highly resistant. Such, indeed proved to be the case.

The blood at our disposal was very small in amount and had been mixed with a small amount of citrate of sodium to prevent coagulation.

From the examination of this citrated blood it was found that one volume of the blood required dilution with two volumes of, at least, a $\frac{N}{250}$ NaCl solution before haemolysis took place. This is a very remarkable result, and would most distinctly point to the presence of some substance conferring a very high degree of immunity to haemolysis on the red blood corpuscles of a patient recovering from blackwater fever. (Normal red blood corpuscles break up when one volume of blood is mixed with two volumes of about a $\frac{N}{60}$ to $\frac{N}{40}$ NaCl solution). This substance must be of the nature of an antihaemolysin, the presence of which would certainly mean the earlier presence of a haemolysin.

ON VARIATIONS OBSERVED IN THE COMPOSITION OF SODIUM GLYCOCHOLATE PREPARED BY DIFFERENT METHODS

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(*Received February 6th*, 1908)

Having had occasion to determine the molecular weight of sodium glycocholate in connection with other work, the results obtained were of so unexpected a character that it was thought worth while to investigate the matter more closely.

The material employed (and referred to above) was Merck's 'sodium glycocholeate.' The method of preparation as carried out by the makers is as follows :—

Ox bile is treated with a solution of lead acetate. The precipitated lead salts are decomposed by sodium carbonate and the residue extracted with alcohol. The alcohol is evaporated off and 'sodium glycocholeate' results.

It is evident that 'sodium glycocholeate' is at least a mixture of sodium glycocholate and sodium taurocholate. The makers themselves state that 'the substance is used for medicinal purposes only and is no chemically pure body.' From the generally accepted view, however, of the relative proportions of the constituents of ox bile, one would expect this preparation to be at least 90 per cent. pure sodium glycocholate. An examination of the substance, however, showed this to be by no means the case.

The empiric formulae and percentage composition of sodium glycocholate and sodium taurocholate usually stated are due to Strecker,[1] and are as follows :—

	Formula	Molecular weight	% N	% Na
Sodium glycocholate	$C_{26}H_{42}NO_6Na$	487	2·87	4·72
Sodium taurocholate	$C_{26}H_{44}NSO_7Na$	537	2·60	4·25

In a preparation of the mixed salts, therefore, one would expect values for the percentage composition to lie between those given in the table, but approximating more closely to those for sodium glycocholate since this is present in much greater proportion than sodium taurocholate.

The 'sodium glycocholeate' having been first tested for absorbed moisture (found 2·6 per cent.) and for the presence of inorganic impurities (*e.g.* sodium chloride and sodium carbonate—which were found to be absent), the following systematic examination was carried out :—

I. Determination of the molecular weight in aqueous solution—
   (*a*) By the lowering of the freezing point.
   (*b*) By the rise of boiling point.

II. Determination of the molecular weight in alcoholic solution by the rise of boiling point.

III. Determination of the percentage of sodium.

IV. Determination of the percentage of nitrogen.

I.—Determination of the Molecular Weight in Aqueous Solution

(*a*) *By the lowering of freezing point of water :—*

Mass of the solvent = 22·5 grams

Mass of 'sodium glycocholeate' in grams	Freezing point (Beckmann therm.)	Δ	Molecular weight
0·0	2·78	—	—
0·1125	2·71	0·07	134
0·5332	2·46	0·32	139
0·8664	2·26	0·52	139

The mean molecular weight in water is therefore 137.

1. Strecker, *Liebig Ann. d. Chem.*

(*b*) *Molecular weight by the elevation of boiling point*:—

Mass of solvent = 26·30 grams

Mass of 'sodium glycocholeate' in grams	Boiling point (Beckmann therm.)	Rise of boiling point	Molecular weight
0·0	1·918	—	—
0·1765	1·940	0·022	158
0·5265	2·009	0·091	114 ?
0·9205	2·049	0·131	140

First and last determination, mean, 149

The values obtained for a molecular weight by the elevation of boiling point when *water* is the solvent can scarcely be taken as conclusive in themselves, owing to the comparative difficulty of carrying out a determination with this solvent. The above results must, therefore, be regarded as surprisingly good corroboration of the more trustworthy determinations obtained by the freezing point method. Since we are dealing with an alkali salt in aqueous solution it is evident that dissociation has taken place, and the above solutions are sufficiently dilute to cause approximately complete dissociation. Assuming the substance to be a mono-sodio derivative we obtain the result that the *undissociated* molecular weight is 278. To check this, a determination was carried out in alcohol.[1] This requires great care in order to get rid of traces of moisture both in the alcohol and the bile salt—for this latter is exceedingly hygroscopic.

II.—Molecular Weight of the 'Sodium Glycocholeate' by the Elevation of the Boiling Point of Alcohol

'Sodium glycocholeate' in grams	Rise of boiling point	Molecular weight
0·2208	0·037	282
0·2820	0·047	283
		Mean, 283

1. Solutions of soaps in alcohol give molecular weights corresponding to non-dissociation. It was therefore thought (and the results justify the assumption) that no dissociation of the bile salt would take place in this solvent.

The good agreement between the results obtained for the substance in the undissociated state in alcohol and that calculated from the determination in aqueous solution, points apparently to the possibility of error in the usually accepted empirical formula and molecular weight.

III.—Percentage of Sodium in 'Sodium Glycocholeate'

The sodium was estimated in the ordinary manner by first incinerating the substance and finally estimating as sulphate.

The following results were obtained :—

'Sodium glycocholeate' in grams	Sodium sulphate in grams	Percentage of sodium
[0·7585	0·1894	8·09]
1·0491	0·2813	8·6
1·0125	0·2702	8·6
	Mean,	8·6

Assuming the substance to be a *mono*-sodium derivative—

A molecular weight of	283	requires	8·12 %	sodium
„ „	487	„	4·72	„
„ „	537	„	4·28	„

The above results seem to afford fairly conclusive evidence in favour of the smaller value for the molecular weight. An alternative hypothesis, however, might be possible. The formulae of the bile acids may be mainly according to those of Strecker, but the acids may be dibasic and hence their salts may contain two sodium atoms, viz., $C_{26}H_{41}NO_6Na_2$ and $C_{26}H_{43}NSO_7Na_2$. This would almost double the sodium percentage, which would be in agreement with that found. Also a disodium salt in dilute aqueous solution would give rise to three ions approximately; thus yielding a molecular weight—$3 \times 140 = 420$. This, although a much higher value, differs considerably from the accepted value. The fact that this is at complete variance with the actual value observed in alcohol renders the hypothesis practically untenable.

To further test the values obtained for the molecular weight the percentage of nitrogen was determined by Kjeldahl's method.

IV.—Nitrogen Percentage

'Sodium glycocholeate' in grams		Percentage nitrogen
0·832	...	1·03
1·087	...	0·90
		Mean, 0·965

Assuming one nitrogen atom in the molecule of salt—

The nitrogen percentage required for molecular weight 283 is 4·94
" " " " 487 " 2·87
" " " " 537 " 2·60

The experimental values for the nitrogen are therefore at utter variance with all the molecular weights, the discrepancy being most marked in the case of the molecular weight actually determined.

All this conflicting evidence can be explained only by assuming that either Strecker's formula is quite wrong, or that there are other substances present whose existence has been overlooked.[1]

In fact *if we assume that over 50 per cent. of the 'sodium glycocholeate' consists of sodium salts of nitrogen-free acids of molecular weight much smaller than sodium glycocholate, the anomalies observed would be accounted for.*

To form an approximate idea of the proportion of true sodium glycocholate in 'sodium glycocholeate' a quantity of the latter was hydrolysed with alkali, and the cholic acid produced was estimated by Lassar-Cohn's[2] quantitative method. The yield was 3 grammes of cholic acid from 8·5 grammes of 'sodium glycocholeate,' the theoretical yield being 6·4 grammes of cholic acid. This points to the percentage of true sodium glycocholeate being about 50 per cent. of the material.

To settle the question, a quantity of ox bile was obtained and from it (1) the bile salts were prepared by Plattner's method, and (2) 'sodium glycocholeate' prepared according to Merck's method. The results of the analyses of the substances prepared by these two methods are given below.

1. Note: It may be mentioned that the presence of sodium oleate was tested for by formation of the lead salt and extraction with ether—negative result.

2. Lassar-Cohn, *Ber. d. deut. chem. Gesell.*, 26, Part I, p. 146 (1893).

(1) The Bile Salt Prepared According to Plattner's Method

This method, as is well known, simply consists in evaporation of the bile on the water bath and extraction of the residue with alcohol, thereby removing the sodium glycocholate and sodium taurocholate.

(*a*) *Determination of the molecular weight of the bile salt thus obtained.*

Mass of solvent (water) in grams	Mass of solute in grams	Δ	Molecular Weight weight
27·70	0·5890	0·15°	262
31·55	0·5830	0·13°	263
		Mean,	262·5

Assuming that the substance is about 80 per cent. dissociated at this dilution in water, we find for the undissociated molecular weight the value 472. This is somewhat low but is in fair agreement with the value 487 required for the sodium glycocholate according to Strecker.

(*b*) *Determination of Nitrogen* (Kjeldahl).

Grams. of substance	N in grams	Nitrogen percentage
1·123	0·02856	2·55

The value required for Strecker's sodium glycocholate is 2·8 per cent.

(*c*) *Determination of the sodium content.*

Substance in grams	Weight of sodium sulphate	Sodium percentage
1·1908	0·1931	5·2

The value required for sodium glycocholate is 4·7 per cent.

This examination of the product obtained by Plattner's method confirms the usually accepted formulae and constitution of sodium glycocholate *when this body is obtained pure.* The small discrepancies noted above can again be explained by assuming the presence in small quantities of that substance (or substances) which appear in large quantities in Merck's preparation.

(2) Analysis of Product Obtained by Merck's Method of Preparation

A quantity of fresh ox bile was treated according to Merck's method, the product yielding the following results on analysis :—

Percentage of Sodium :

Substance in grams	Sodium sulphate in grams	% Na
1·1412	0·2152	6·11
1·0097	0·1902	6·10
	Mean,	6·1

Percentage of Nitrogen :

Substance in grams		Percentage nitrogen
0·4426	...	2·66
0·8979	...	2·40
	Mean,	2·53

We have here again in the case of the sodium content the discrepancies already noted. The values for nitrogen, however, as will be seen, lie not far from those for pure sodium glycocholate. There can be little doubt but that we are in all cases dealing with the same contaminating substance, though the amount of this substance appearing in the final product depends on the conditions of manipulation.

The most unexpected thing about this substance is the exceedingly large amount of it evidently present in ox bile ; especially when one remembers that the exhaustive work of Lassar-Cohn[1] and others point to the fact that in ox bile the sodium glycocholate and sodium taurocholate are present in much greater proportion than all the other salts together. For example, after hydrolysis of the glycocoll and taurine derivatives, Lassar-Cohn[1] found that ' 100 litres of ox bile yield 4,790 grammes of cholic acid and 405 grammes of other acids.'

1. Lassar-Cohn, *Ber. d. deut. chem. Gesell.*, 26, Part I, p. 146, (1893).

The important question is, of course—what is this hitherto undetected substance or substances? In this connection it may be mentioned that an attempt was made to ascertain whether there was any formation of double salt between the pure sodium glycocholate and some of the precipitants or products of precipitation. Thus, a quantity of the material obtained by Plattner's method was dissolved in water and treated with excess sodium acetate. Lead acetate was then added, and the product decomposed with sodium carbonate and evaporated to dryness. In the presence of sodium acetate it was thought that possibly a double salt of the form ($C_{26}H_{42}NO_6Na + C_2H_3O_2Na$) might have been produced and removed by the final extraction with alcohol. The substance obtained, however, proved to be simply sodium glycocholate, an analysis of the material yielding 5·0 per cent of sodium.

Referring to Merck's preparation of 'sodium glycocholeate,' the analyses already given allow one to calculate the mean molecular weight of the substance (or substances) whose presence has been suspected.

Thus assuming that the unknown body is nitrogen free, the determination of nitrogen recorded leads at once to the conclusion that Merck's preparation consists of 35·7 per cent. sodium glycocholate and 64·3 per cent. unknown substance (or substances).

Now if

M = the undissociated molecular weight of pure sodium glycocholate = 487

M' = the mean undissociated molecular weight of the mixture = 282

m = the mean undissociated molecular weight of the unknown substance (or substances)

it follows that:

$$\frac{100}{M'} = \frac{35{\cdot}7}{M} + \frac{64{\cdot}3}{m}$$

whence $m = 227$, and as this is a sodium salt or salts (presumably a mono sodium derivative) the mean molecular weight of the acid (or acids) is 205.

In the saturated fatty series of acids the following members occur—

Lauric acid	$C_{12}H_{24}O_2$	molecular weight 200
Tridecylic „	$C_{13}H_{26}O_2$	„ „ 214
Myristic „	$C_{14}H_{28}O_2$	„ „ 228

If we are dealing with a single substance in the case of the unknown salt the value obtained for the molecular weight points strongly to the presence of sodium laurate. It is much more probable, however, that we are really dealing with more than one body. In this connection one may mention the acids whose salts are present in ox bile according to Lassar-Cohn,[1] viz., glycocholic, taurocholic, stearic, palmitic, oleic, myristic and some 'amorphous acids.' According to Lassar-Cohn, as already stated, the first two acids are present in amount about 10 times that of the remaining acids added together. It is very suggestive, however, that myristic acid should have been found as a constituent of ox bile, although Lassar-Cohn's analyses point to its being present in extremely small quantity.

Myristic acid, as already stated, has a molecular weight of 228, which is not so very far removed from the mean value 205 of the unknown constituent. And indeed this mean value would be readily realised by myristic acid containing a small proportion of some acid of much lower molecular weight, as for example valeric acid (molecular weight 102), or caproic acid (molecular weight 116), both of which are known to be produced in animal metabolism.

Summary

The result of the foregoing investigation has been to confirm Strecker's formula for sodium glycocholate when this is obtained in the pure state. At the same time, evidence is adduced to show that in the ordinary methods of extraction of mixed bile salts (*i.e.* sodium glycocholate + sodium taurocholate) one does *not* always obtain a mixture consisting entirely (or almost entirely) of these two salts.

1. Lassar-Cohn, *loc. cit.*

The resulting product appears to contain as well, varying amounts of the sodium salts of nitrogen-free fatty acids of very much smaller molecular weight which may, in certain cases, be present to the extent of over 50 per cent. of the 'mixed bile salt' preparation.

It is suggested that sodium myristate is the chief representative of these lower fatty salts, this body being probably present in ox-bile to a very much greater extent than the analyses of Lassar-Cohn seem to show.

NOTES ON THE ACTION OF ATROPINE, HYOSCYAMINE, HYOSCINE, SCOPOLAMINE, DUBOISINE, AND DATURINE

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From the Physiological Laboratory of the University of Manitoba

Communicated by Professor SWALE VINCENT, M.B., D.Sc.

(*Received March 8th*, 1908)

The primary object of the present research was to test the efficiency of atropine as a restorative in poisoning by chloroform or other anaesthetic, or as a precautionary measure before its administration. The use of atropine in one or other of these ways has been frequently advocated.[1]

At the outset of my series of experiments a somewhat striking discrepancy was noticed between the statements in the text-books and the results actually obtained, even as regards the most readily observable effects upon the heart and blood vessels; thus it was deemed advisable to perform an extensive series of experiments devoted to the physiological effects of atropine and its allies upon the heart, respiration and circulation, apart altogether from the question of benefit or otherwise in the administration of anaesthetics.

Atropine and the allied drugs mentioned in the title are generally supposed to be isomeric with each other, or very closely allied. There is a close relationship between these substances as regards not only their chemical structure but also their physiological action, and as far as my experiments are concerned, it is impossible to detect any difference between them. In regard both to their general physiological effects and to the question of the rapidly induced tolerance or immunity described later, all these drugs may be considered as

1. Dixon, *Manual of Pharmacology*, p. 79, London, 1906.

identical.[1] My experiments, however, have been restricted to what we may call gross effects upon the heart, circulation and respiration. The effects on the central nervous system, nerve endings, secretion, and excretion have not been specially examined in the present investigation, and it is, of course, possible that in some of these spheres of action there may be individual differences between the different drugs.

The question of the value of adrenalin as a restorative to the circulatory system will be incidentally discussed in the body of the paper.

I have performed in all more than fifty experiments—two on cats, the rest on dogs. Chloroform, ether, or the A.C.E. mixture were the anaesthetics used. In some few cases curari has been used in addition. The blood pressure has been taken from the carotid artery, and the injections made into the saphenous or femoral vein. A glass plethysmograph was used for recording changes in the volume of the limb, and for similar changes in the intestinal wall an air oncometer was used, each of these being connected with a piston recorder. The method described by Oliver and Schaefer[2] was employed for recording the effects upon the heart. A hook is caught in the epicardium of the auricle, and another in that of the ventricle. From these threads pass over pulleys moving on a horizontal axis; the threads then pass vertically downwards to be attached to long elastic levers of steel. To the ends of the levers writing points are attached.

Physiological Effects

One of the most familiar, and at the same time most striking, actions of atropine is paralysis of the peripheral terminations of the vagus in the heart. It would naturally be expected that this effect, like section of the vagi, since it cuts off the tonic inhibitory influence

1. For information on the chemistry of these substances see Schmiedeberg, *Pharmakologie*, 1906; Tomasini, *Atti dell. R. Accad. dell. Scienz. Med.*, Palermo, Ann. 1896; O. Hesse, *Leibig's Annalen*, Vol. CCLXXVII, 304, 308; O. Hesse, in *Apotheker Zeitung*, 1895; Raehlmann, *Semaine Medicale*, 1893; Bokenham, *B.M.J.*, Vol. II, p. 597, 1894; T. R. Pooley, *Can. Lancet*, Jan., 1895; Sharp, *Practitioner*, Jan., 1894; Pavloff, *St. Petersberg Med. Chi. Diss.*, 1889-1890; *Year Book of Pharmacy*, 1880, 1881, 1882, 1885, 1892, 1893, 1894; *Pharm. Journ.*, 1878.

2. *Journ. of Physiol.*, Vol. XVIII, p. 256, 1895.

of the nerve centre, would exercise an augmentor effect upon the heart, and raise the blood pressure. This is, in fact, usually stated to be the case. Thus Dixon[1] says: 'In mammals small injections of atropine produce the same result: this paralysis of the peripheral vagal terminals, like section of the vagi, cuts off the tonic inhibitory influence of the centre, and the heart is quickened. The increased rate will naturally only occur in those animals in which there is some tonic central effect. Thus the quickening is decided in dogs, and little in cats, whilst in man it varies with the age and disposition, but is usually greatest between the ages of twenty-five and forty. In children under two months atropine causes no quickening, and it has also little effect in old age.' This author also suggests that atropine may directly stimulate cardiac muscle, and refers to a vaso constriction as a result of the action of the drug on the medulla. 'Blood pressure rises mainly as a result of this vaso constriction . . . the pressure also tends to rise on account of the quickened heart . . . . . constriction of vessels is pronounced only in the splanchnic area.' Schmiedeberg[2] likewise states that atropine, in men and dogs, quickens the heart and raises blood pressure, but admits that a subsequent effect is paralysis of the heart.[3] Sollmann[4] states that the blood pressure is scarcely altered, but there may be a slight rise from stimulation of the vasomotor centre; this stimulation is always slight, and may be entirely absent. It is replaced by vasomotor depression rather early. Large doses depress the vasomotor centre profoundly, so that the pressure falls very low while the heart is still beating. Still larger doses paralyse the heart muscle as well. Sollmann is the only author I am acquainted with who mentions this lowering of blood pressure at all. Thus Dixon states that there is a rise of blood pressure owing to vaso constriction; Sollmann that there is at first a slight rise owing to vaso constriction, and later a pronounced fall owing to vaso dilatation. But my own experiments

1. *Op. cit.*
2. *Op. cit.*
3. This secondary effect is also mentioned by Pouchet, *Leçons de Pharmacodynamie*, Paris, 1901.
4. Sollmann, *Text-book of Pharmacology*, Philadelphia, 1906.

furnish no evidence of any action whatever on the vasomotor system. I have been unable to find any original papers giving details of experiments upon animals, with tracings of the blood pressure.[1]

In my series of more than fifty experiments on dogs *I have never observed any rise of blood pressure upon the injection of atropine into the circulation.*[2]

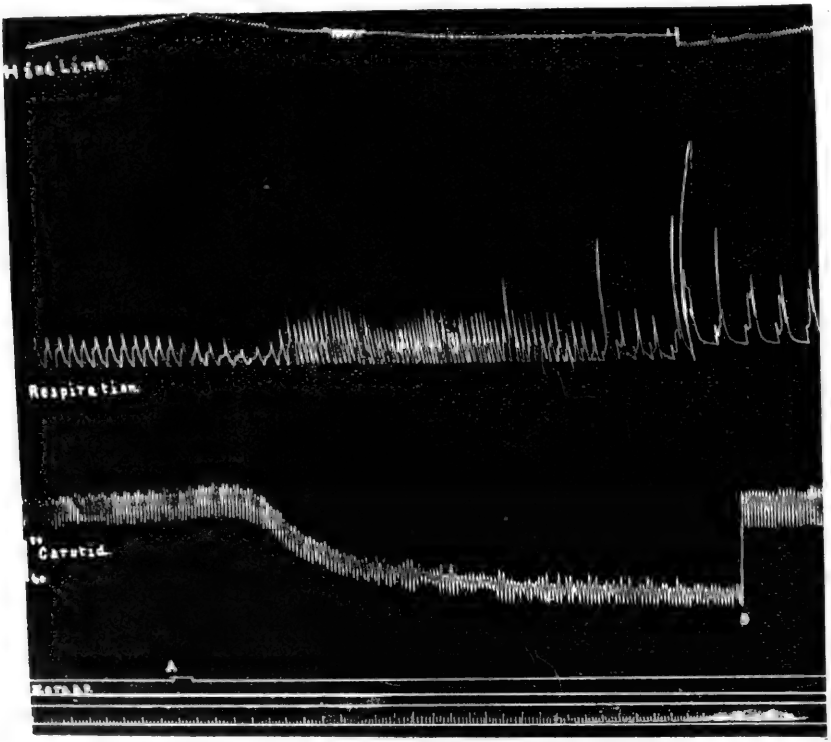

FIGURE 1.—Dog, 10·5 kg. Chloroform. No anaesthetic had been given for half an hour before the injection, at A, of 0·4 of a gram of atropine. The animal had previously received, in increasing doses, commencing with 0·7 of a mg. of hyoscine, 50 mg. of hyoscine and a 0·5 of a gram of atropine. The blood pressure falls and returns to normal in 7·5 minutes. At B, the kymograph was stopped for 5 minutes. Respiration was first quickened, then, as the blood pressure returns to the normal, it is markedly slower but deep. The limb follows passively the blood pressure. Scale, half.

1. The literature, however, to which I have access is limited.

2. Professor Vincent informs me that so far as his memory serves him he has never seen a rise of blood pressure on the administration of atropine.

This applies to the allied drugs mentioned in the introduction. The drugs have been tested under very varying conditions as to dosage, amount of fluid injected, temperature of fluid injected, and rate of injection. In all cases, when any effect whatever has been produced, this has been in the direction of a fall of blood pressure. In small doses this is slight and transient;[1] in large doses marked and long continued—sometimes for an hour. In some cases, however, even after large doses, recovery is fairly rapid. (See Fig. 1.)

In many cases, it is true, the tracings show a slight preliminary rise of blood pressure. This, generally followed by a much more pronounced and significant fall, is, I have convinced myself, simply due to the injection of the fluid in which the drug is dissolved, since an injection of an equal quantity of normal saline solution has been always found to induce a rise of pressure similar in character, and of equal magnitude. (Fig. 2.)

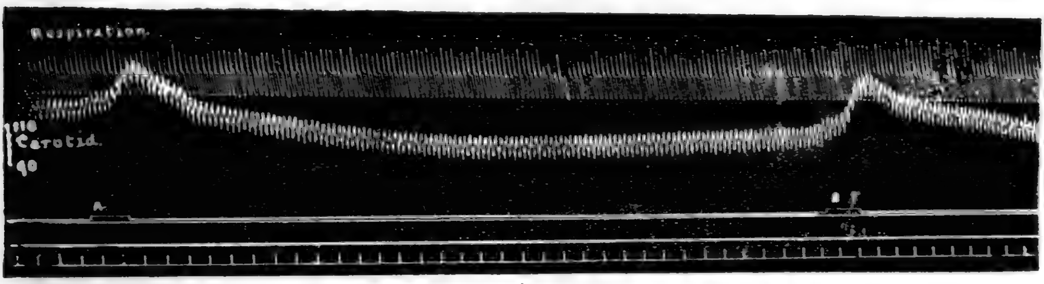

FIGURE 2.—Dog, 5·3 kg. A. C. E. At A, 0·5 of a mg. of atropine was given in 10 c.c. saline solution. At B, 10 c.c. saline solution only was injected. The same initial rise of blood pressure occurs in both instances. Time intervals, 5 secs.

Of course, the effect of atropine on the blood pressure has almost always been recorded in animals already under the effects of other

1. A possible criticism of my results would be that doses small enough had not been tried, and that the initial increased rate of heart beat and concomitant rise in blood pressure, whether due to this or to vaso constriction, had been overlooked. Every precaution has been taken to avoid this error. No dose, however small, has in my dogs produced the slightest rise of blood pressure. In many cases so small was the amount of drug injected that no effect whatever was produced other than that due to the fluid administered, which point has been very frequently tested by control injections of the same quantity of normal saline solution.

drugs. Thus the animal has been under the effects of chloroform, ether, or A.C.E. mixture when the first dose of atropine, hyoscine, etc., has been injected, and in the experiment in which records of auricle and ventricle were taken, curari was administered in addition.[1] On the other hand, many of the larger doses have been given when the animal has not had any anaesthetic for the previous half hour, the atropine already used sufficing to maintain an unconscious condition.

Effect upon the Heart.—Contrarily to the usually accepted view, it has been found that in dogs atropine has only at most a very slight and temporary effect in the direction of augmentation of the heart beats; the chief effect is a diminution in the extent of movement as revealed by the heart levers. (See Figs. 3 and 4.)

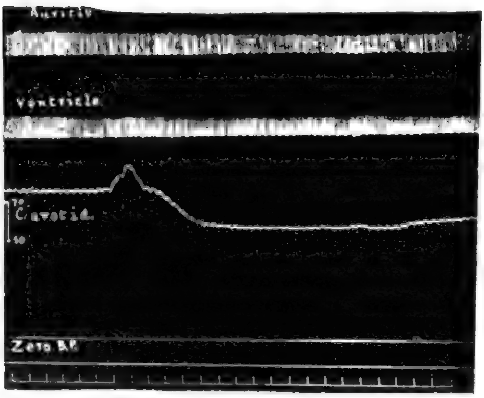

Fig. 3

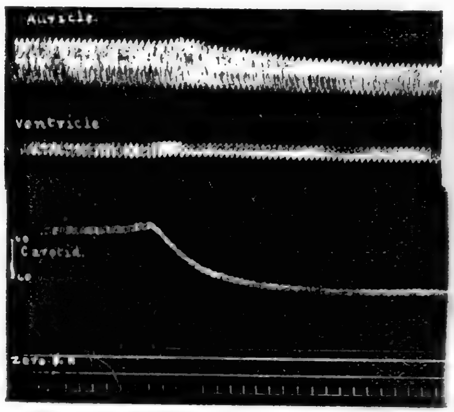

Fig. 4

Figure 3.—Dog, 5·2 kg. Ether. 1 mg. of atropine has the usual effect on carotid pressure. There is slight diminution in extent of movement of auricle and ventricle. Time intervals, 5 secs.

Figure 4.—Dog, 11·5 kg. Ether, curari; artificial respiration. Atropine, 1 mg. Very slight temporary increase of frequency of heart beat, followed by diminution in extent of movement, as shown also in Figure 3. Time intervals, 5 secs.

1. In one experiment I have reduced this objection to a minimum by employing only just sufficient anaesthetic for the carrying out of the preliminary surgical proceedings. The animal was then allowed to recover from anaesthesia, and atropine in a small dose injected into a vein. The effect in this case, as usual, was a *fall* and not a *rise* of blood pressure, and the heart was weakened. (See Fig. 10.)

Mode of Action of the Drugs.—The levers of the piston recorders connected with the limb plethysmograph and the intestinal oncometer always fall on the injection of the drug. The limb and intestinal tracings, in fact, passively follow that of the blood pressure, and frequently show the same preliminary rise as does the blood pressure. (Figs. 5 and 6.)

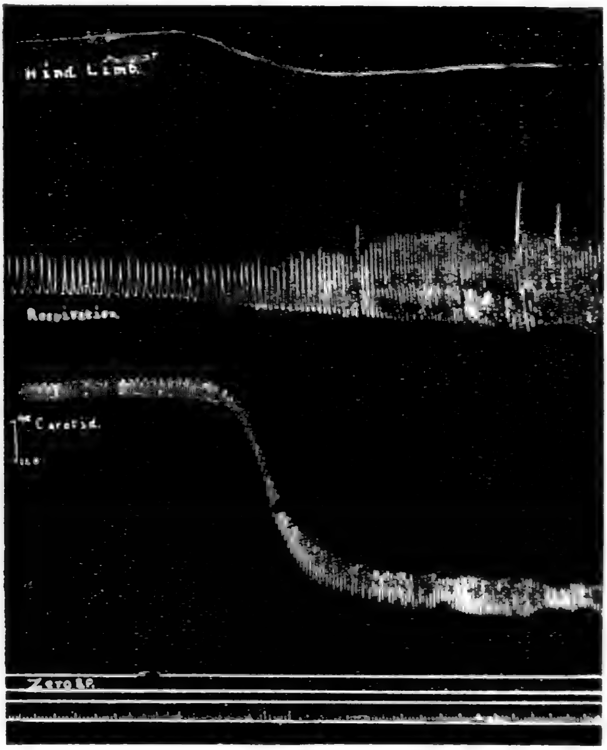

FIGURE 5.—Dog, 10·5 kg. Chloroform, atropine, 0·2 of a gram. The animal had previously been given increasing doses of hyoscine and atropine. The blood pressure was markedly lowered but the animal recovered. The heart was slowed, respiration was increased in frequency and depth and rendered more irregular. The volume of the limb follows the blood pressure. The heart beat is less frequent and more irregular, as indicated by the movements of the mercury in the manometer. The slowing of the heart beat is shown by the increased excursions of the mercury, which are shown most distinctly at the lowest part of the curve. Such increased excursions are frequently misinterpreted as meaning increased force of heart beat.

This must be interpreted as meaning that owing to enfeebled heart's action blood is drained from both the somatic and splanchnic

areas ; there cannot be active vaso constriction, or there would be a rise of blood pressure, and we have seen that there is never any evidence of vaso dilatation.

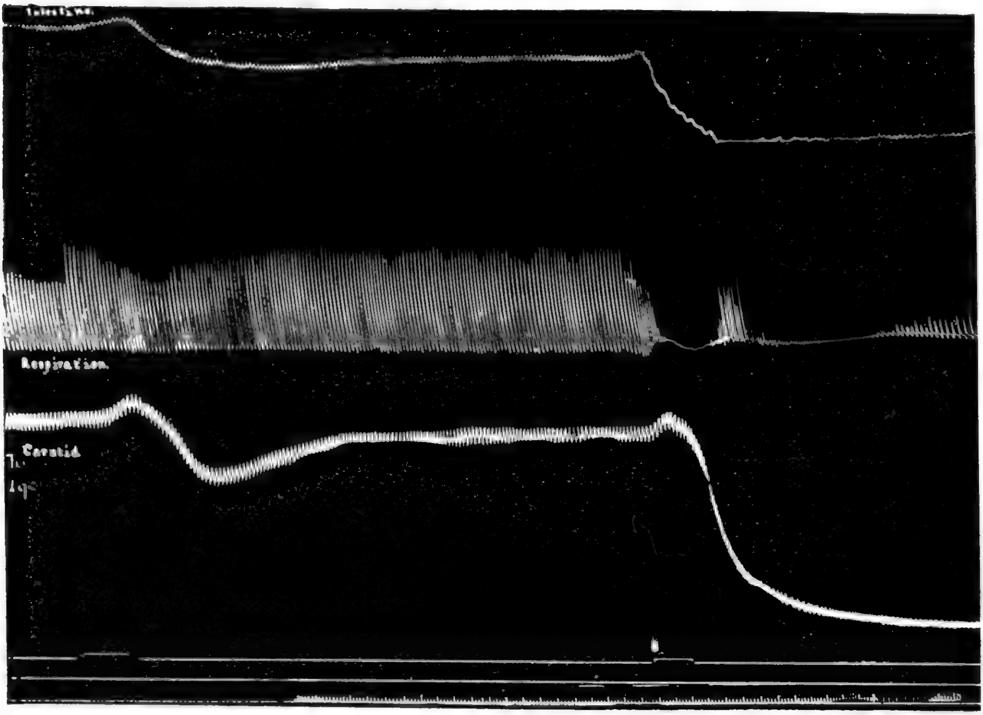

FIGURE 6.—Dog, 7 kg. At A, 8 mg. of atropine is administered in 10 c.c. saline solution. The animal has already had 7·5 mg. of atropine in divided doses. At B, 20 mg. of atropine, in 10 c.c. saline solution, proved fatal in 10 minutes. Intestinal volume follows the blood pressure after both doses. The slight preliminary rise of blood pressure is usually obtained and is due to the quantity of fluid injected, as it can be invariably reproduced by the injection of 10 c.c. saline solution. The short lines of the time marking show seconds, the long lines 5 seconds.

It would appear that rather too far-reaching deductions have been drawn from the action of atropine in cutting out the inhibitory control of the vagus. Since cutting the vagi always *raises* the blood pressure, while the administration of atropine always lowers it, in the dog at any rate ; and since, on injection of atropine, the volume of both limb and intestinal wall always follow passively the blood pressure, we must conclude that atropine acts upon the heart in a manner quite

different from that of section of the vagi. (Fig. 7.) It seems, in fact, that with atropine, although the vagus inhibition is removed, there is a much more powerful effect acting upon the circulation in an opposite sense, namely, a paralytic effect on the heart muscle itself.

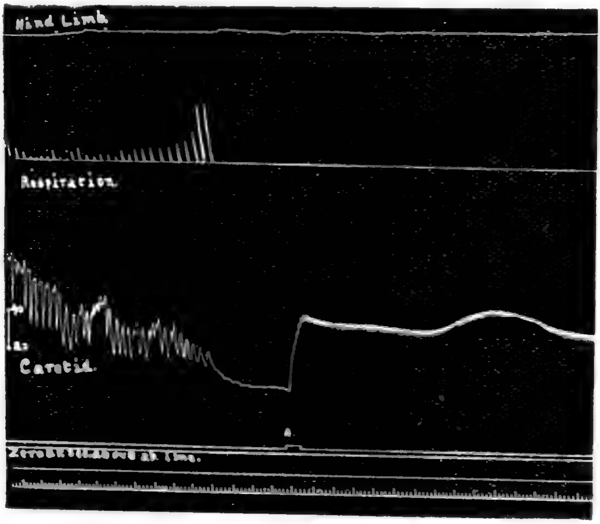

FIGURE 7.—Dog, 5·2 kg. Overdose of chloroform. At A, both vagi were cut simultaneously; sudden rise of blood pressure occurs. Respiration, which had stopped, was not re-established, and blood pressure gradually falls again. The animal died in about 6 minutes.

THE EFFECTS OF RAPIDLY REPEATED DOSES OF THE DRUGS

It is well known that a very marked tolerance to atropine as well as to other drugs can be established both in animals and man by gradually increasing the dosage over a period of days, weeks, or months. It is, however, somewhat surprising to find that within the limits of time occupied by a single experiment a dog can be brought to withstand, manifesting only a comparatively slight reaction, a dose which, if administered at the beginning of the experiment, would have been certainly and quickly fatal; nay, further, in many cases even a large multiple of this. This rapid immunity or tolerance to the poisonous effects of a drug is not referred to in any of the books or papers to which I have had access, though the phenomenon is so striking that one can scarcely believe it has escaped the notice of pharmacologists.

By commencing with small doses and gradually increasing their size, animals of 5 to 12 kg. body weight have, in the course of an experiment lasting one and a half hours, been rendered so immune to the ill effects of the drug as to tolerate as large a quantity as 0·4 gramme injected intravenously (Fig. 1) without other apparent ill effects than a lowering of blood pressure, from which recovery gradually takes place.[1]

The commencing dose varied from 0·1 mg. to 0·5 mg. of atropine, hyoscine, etc., an injection being given every three to ten minutes afterwards until the animal succumbed. In some cases 0·5 mg. as an initial dose caused death rapidly, but in others 1 mg., or even 2 mg., could be used as an initial dose without a fatal result, the animals showing the same idiosyncrasy as the human subject in this respect. In all cases where the initial dose failed to kill each successive dose was doubled or trebled until on many occasions 0·4 of a gramme of the alkaloid was given. From this latter dose animals have recovered without the intervention of any restorative measures; double this amount has been given, and the animal kept alive by artificial respiration, the blood pressure, which was very low after the injection, rising almost to the normal. But immediately on stoppage of the artificial respiration the pressure would fall and death ensue.

It does not make any difference which of the drugs, atropine, hyoscine, hyoscyamine, duboisine, daturine or scopolamine, are used to commence with in the process of obtaining this tolerance; any one can be used in increasing doses; then, when the dose has become large, an increased dose of any of the others given with no different result than would be obtained were the first drug continued—each drug immunising the animal from all the rest of the series. Further, the serum of an animal which has been rendered immune to large doses of these drugs will, if injected into another animal, confer an immunity on it.

Thus a first dose of 0·5 mg. of duboisine injected into a dog of 6 kg. weight produced rapid fall of blood pressure and death. (See

1. It is impossible to make any definite statements as to the permanent effects of such a dose, as in this and all other cases the animal was killed under the anaesthetic.

Fig. 8.) Later, blood serum,[1] obtained from an animal which had previously had large doses of hyoscine and atropine, was injected into another dog 6 kg. weight. The serum, amounting to 80 c.c., was given in eight doses by means of a 10 c.c. syringe. A very slight effect was noticed with the first and second dose, given at intervals of three minutes, and between the second and third doses; the remaining six doses were given as quickly as the instrument could be filled, and no effect was observed. 12·5 mg. (twenty-five times the dose which killed the same weight of dog mentioned in Fig. 8) of duboisine were injected about ten minutes afterwards with practically no effect, as seen in Fig. 9.

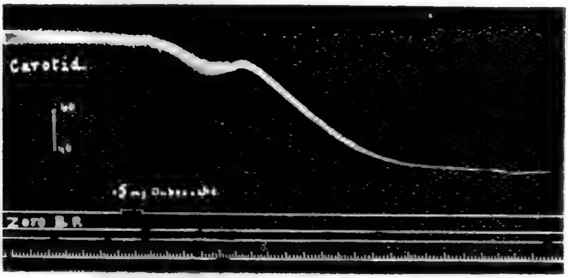

FIGURE 8.—Dog, 6 kg. Duboisine, 0·5 mg. caused death in two minutes. This was a primary dose.

From this experiment it would be rash to conclude that anything of the nature of an antitoxin had been formed. The first dose (10 c.c.) of serum from the drugged animal had the effect of cutting out the vagus action in the second animal, and it is probable that this serum contained therefore considerable quantities of the drug injected. This has an important bearing upon one theory of toleration, rendering it clear that since the serum contained the drug in an active form the tolerance established in the first dog could not be due to rapid elimination.

1. Blood was drawn from an animal which had had large quantities of hyoscine and atropine, and after standing twenty-four hours the serum was drawn off with a pipette in the usual manner.

Action on the Respiratory System

It is well known that the first effect of atropine upon the respiration is to increase both the frequency and extent of the movements. (Figs. 1, 5, 9.) Subsequently, however, the respiratory centre is paralysed. (Figs. 6 and 7.) I have been able to confirm the statement made by Reichert,[1] and quoted by Sollmann,[2] that an animal may recover from many times the minimal fatal dose if artificial respiration be maintained. This power of recovery on the part of the respiratory centre is of supreme practical importance in dealing with cases of poisoning by this drug.

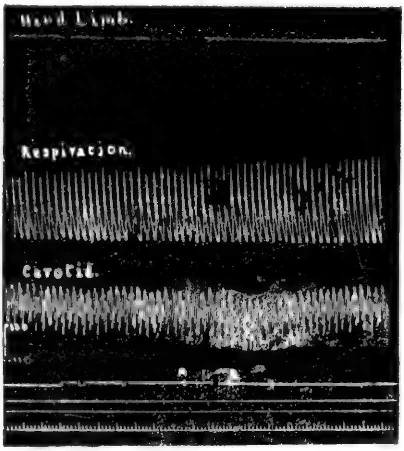

Figure 9.—Dog, 6 kg. 80 c.c. of blood serum from a dog (which had had altogether 65 mg. of hyoscine and 1·64 grams of atropine in an experiment extending over nearly two hours) was administered. At A, 12·5 mg. of duboisine was administered with practically no effect. Previously (Fig. 8) 0·5 mg. ($\frac{1}{25}$ the dose) given as a first injection, without a preliminary one of blood serum, killed a dog of the same weight in two minutes.

'When the blood pressure has been depressed by an overdose of chloroform, section of the two vagi, by cutting off the medullary effect, will release the heart; the beat will once again recover its normal character, and the blood pressure will bound up.'[3] A natural

1. Reichert, *Phila. Med. Jour.*, Jan. 19th, 1901.
2. Sollmann, *op. cit.*
3. Dixon, *op. cit.*

inference would be that atropine, by cutting off the tonic effects of the vagus, would have a similar effect. This, however, has not been the case in my experiments.[1] In chloroform poisoning, just as in the normal condition, atropine does not raise the blood pressure, but lowers it. I have not found the slightest benefit to accrue when atropine has been administered to an animal whose circulation is depressed with chloroform or other anaesthetic.

The use of atropine prior to the administration of chloroform has been strongly advocated and no less strongly opposed by various writers, *e.g.*, Brodie and Crouch,[2] J. Harley[3], Dastre[4], Pitha[5], Fraser[6], Brown-Sequard[7], Dastre and Morat[8], Schäfer[9], Schäfer and Scharlieb[10], Hewitt[11], some of whom question the great danger of primary inhibition of the heart through excitation of the vagus, and also the benefit supposed to be derived from a preliminary dose of atropine.

The dose required to eliminate vagus action in dogs varies greatly per kg. of body weight in different individuals. This variability, we know, exists to as great an extent in the human subject. In view of the fact that we have no convenient means of testing vagus condition in the human subject, it follows that after administering a dose of atropine previously determined upon we are in the dark as to whether we have obtained the desired effect. Should we invariably give a large dose, disagreeable and possibly fatal results might ensue; numerous cases have been recorded where small doses have produced ill effects.

1. I have not invariably found benefit from cutting the vagi, although often, as Dixon states, it restores blood pressure. In the case of the animal whose tracing is reproduced in Fig. 7, although a sudden rise of blood pressure took place, respiration was not restored; the blood pressure again fell, and death rapidly ensued.

2. *Trans. Soc. Anaesth.*, Vol. VI, pp. 70 and 81, 1903.

3. *Brit. Med. Jour.*, Vol. II, p. 320, 1868.

4. *Soc. Biol.*, p. 242, 1883.

5. Pitha, 1861. Quoted by Schäfer from Dastre.

6. *Brit. Med. Jour.*, Vol. II, p. 715, 1880.

7. Brown-Sequard (*C. r. Soc. Biol.*, p. 289, 1883).

8. Dastre and Morat (*Lyon Med.*, 1882, and *C. r. Soc. Biol.*, pp. 242 and 259, 1883).

9. Schäfer, *Brit. Med. Jour.*, Vol. II, p. 620, 1880.

10. *Trans. Royal Soc. Edin.*, p. 333, 1904.

11. *Anaesthetics and their administration*, pp. 230, 259, 503, 1907.

Harley advocates 0·01 to 0·025 grain, and Dastre 0·0015 gramme (0·023 grain). These doses would certainly cause serious symptoms in some subjects, and would probably cause discomfort in nearly all, and in spite of their size we have no guarantee that the quantity is sufficient to abolish vagus action. It may also be argued that after an anaesthetic the sleepiness and depression are such that the dose of atropine would be eliminated before consciousness completely returned. This would only be the case after prolonged anaesthesia. After short operations, where the anaesthetic is skilfully administered, consciousness returns with comparative rapidity, while the effects of atropine last twelve to twenty-four hours; and in these short cases the atropine is quite as necessary as in cases of prolonged anaesthesia if, as stated, the danger to be chiefly averted is that of primary cardiac inhibition.

As regards myself, 0·01 grain administered hypodermically to test the effect on blood pressure produced very disagreeable results; headache, defective vision, dryness of the fauces, and a slight incoordination of the muscles, particularly of the lower limbs, being the prominent symptoms. Blood pressure which was taken previously for three consecutive days at the same hour with Janeway's instrument was recorded every five minutes after the injection at first, then later every ten minutes. It showed a gradual fall until it was reduced by 20 mm. of mercury. Dr. ——, who was treated in the same manner, but only took 0·0067 grain of atropine, had less pronounced symptoms, but still sufficient to cause considerable discomfort, and a very slight change of blood pressure, which was lowered by 10 mm. of mercury.

Among the more recent advocates of the use of atropine, Professor Schäfer must be specially mentioned. His method was to inject atropine *prior* to the administration of the anaesthetic, and hypodermically. In my experiments this has not been done. The atropine has been injected into a vein *during* the actual administration, and at various stages, and with the blood pressure at different levels. So far as my experiments go they do not support the view that the atropine is beneficial.

So far as I can conclude from my experiments, adrenalin would be of distinctly more benefit, though, since its effect is transitory, as a rule frequent small doses must be administered in order to be of benefit. If the heart has not completely stopped, adrenalin causes more active contractions[1] and consequent rise of blood pressure, which a few doses at short intervals may make permanent. If the heart has ceased absolutely, I have never obtained any result. This restoration of heart beat may be obtained after the heart movements can no longer be felt through the chest wall, but when, if the chest wall is opened, small movements can still be observed.

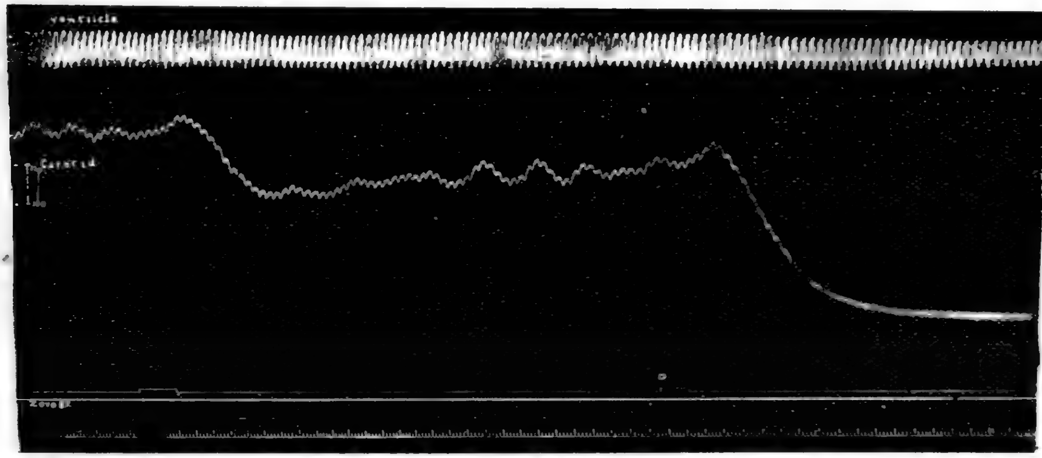

FIGURE 10.—Dog, 7·2 kg. Ether, atropine. At A, 0·02 gram causes, after a slight preliminary rise, a fall in blood pressure and slowing of heart with diminution of the force of the beats. At B, 0·08 of a gram was given with similar, but more pronounced, results.

Summary

1. In dogs atropine, hyoscine, hyoscyamine, scopolamine, and daturine produce, whether in small or large doses, a lowering of the blood pressure.

2. The volume of a limb or portion of intestinal wall always becomes diminished concomitantly with the fall of blood pressure.

1. The rise with adrenalin is due also to stimulation of vasomotor nerve endings in various parts of the body.

3. The conclusion seems justified that although these drugs eliminate the tonic inhibitory action of the vagus, they have a simultaneous action on the heart substance, diminishing the output. This paralytic effect upon the heart is shown also by direct experiment upon this organ.

4. By frequent administration of increasing doses of these drugs an animal may be brought into a condition of tolerance within one or two hours, so that at the end of this time it will withstand (with comparatively slight reaction) very many times the dose which would have been fatal at the beginning of the experiment.

5. In small doses the respiration is quickened and rendered deeper. In large doses it is often paralysed immediately.

6. The present series of experiments has not yielded results which would tend to encourage the use of atropine in chloroform poisoning. Adrenalin seems to be of much more, though limited, utility.

Note added February 6th, 1908

In consequence of some criticism of the preceding observations, which have been privately offered, I have performed a further series of experiments.

The first point in which my results differ from those of the majority of experimenters is that in the dog certainly, and probably also in other animals, a dose of atropine sufficient to produce any effect at all upon the blood pressure does not raise it, but invariably lowers it. This can only mean that the increased force and frequency of the heart's action, which must be brought about by the mere fact of eliminating vagus action, is counteracted even in minimal doses by another effect of atropine, viz., a depressant effect. In other words, the effect of the administration of atropine is something other than a simple cutting out of the vagus.

On this point I have performed a series of ten additional experiments on dogs, and find myself totally unable, under any circumstances whatever, to raise the blood pressure of the animal. The

attempt has been made on a dog without any anaesthetic as well as under the influence of chloroform, ether, the A.C.E. mixture, morphia and curari, and further with the blood pressure at various levels. In all cases when the dose has been sufficient to produce any effect at all there has been a fall and not a rise of blood pressure. It is scarcely necessary to state that account has been taken only of the initial injection in any one animal, since it has never been supposed that atropine would raise the blood pressure after the vagus terminals have once been paralysed. In my experiments 0·5 c.c. of 1-10000 solution per kg. of body weight has usually been found to be the smallest dose which will paralyse the vagus terminals,[1] and this dose never raises the blood pressure.

The second point of interest in the above communication is that during the course of a single experiment lasting two or three hours a tolerance to the action of atropine may be established. It is not pretended that the large doses mentioned in the body of the paper would be completely recovered from, but that they produce effects which are exceedingly small as compared with effects which would be produced at the beginning of the experiment. This general result has been confirmed in ten fresh experiments.

1. The dose varies considerably in different animals, some requiring less, and some a greater dose. This, however, is about the average.

A NOTE ON THE DISTRIBUTION OF THE SALTS IN HAEMOLYSIS

By ALBERT WOELFEL, M.D.

From the Hull Physiological Laboratory, University of Chicago

Communicated by Professor G. N. Stewart

(*Received March 26th*, 1908)

In his extensive studies[1] of the comparative electrical conductivities of normal blood laked by various means, Stewart has shown that complete liberation of the haemoglobin can be produced in blood and in artificial suspensions of erythrocytes without any change or with only a slight increase in conductivity. This is the case when what he calls the less violent haemolytic agents (freezing and thawing, heat, foreign serum, strictly minimal doses of saponin, etc.) are employed. When the laked blood is subsequently exposed to one of the more energetic haemolytic agencies (water, supraminimal doses of saponin, etc.) a marked increase in the conductivity is produced. The total increase is sensibly the same whether the haemolysis has been effected in two stages, first by a gentle and then by a more violent agent, or in one stage by the original and more energetic action of a body of the second group. The action of the more violent haemolytics when consecutive to that of the less violent, is an action on the shadows or ghosts of the corpuscles already deprived of haemoglobin which reduces them to the same condition as that produced directly by the more energetic haemolytics acting alone. It is not due to the

1. *Jour. of Phys.*, Vol. XXIV, p. 211, 1899; *Ibid.*, Vol. XXVI, p. 470, 1901; *Jour. of Exp. Med.*, Vol. VI, p. 257, 1902; *Jour. of Med. Research*, Vol. III, p. 268, 1902; *Amer. Jour. of Phys.*, Vol. IX, p. 72, 1903.

breaking up of (hypothetical) compounds of colloids and electrolytes in the extracorpuscular liquid[1] of the laked blood.

This phenomenon is of interest not only in relation to the mechanism of haemolysis but in relation to the physical chemistry of cells in general, since, as Stewart[2] has pointed out, and as has been more recently insisted on by Overton and others, it is a common property of living cell 'envelopes'[3] to be relatively impermeable to the ions with which they are normally in contact.

In the interpretation of the alterations of conductivity in laking the following points must, according to Stewart, be taken account of :

1. The exit of haemoglobin from the corpuscles (supposing it to take place without interchange, on the whole, of water or electrolytes between the serum and the stromata, and without change in the permeability of the envelopes or stromata to the ions) would depress the conductivity of the laked blood by diminishing the conductivity of the intercorpuscular liquid to a greater extent than would be compensated by the diminution in volume of the badly conducting corpuscles.

2. Apart from the depressing influence of liberated haemoglobin, the exit of electrolytes from the corpuscles would increase the conductivity of the intercorpuscular liquid and, therefore, of the laked blood, provided that the volume of the corpuscles is not increased by the passage of water into them.

3. The exit of water from the corpuscles unaccompanied, on the whole, by electrolytes would cause an increase in the conductivity

1. The extra- or intercorpuscular liquid is a convenient term for the liquid in which corpuscles or ghosts are suspended. In unaltered blood it is, of course, the serum.

2. *Journal of Physiology*, Vol. XXIV, *loc. cit*, 1899.

3. *Note by G. N. S.*—In this paper the question of the precise physico-chemical action produced by the various haemolytics or their precise point of attack in the corpuscle has not been entered into. Moore and Roaf (this Journal, Vol. III, p. 55, 1907) have recently published an interesting paper in which they argue against the view that the difference in the inorganic constituents of corpuscles and plasma can be accounted for by the existence of a 'membrane' variously permeable to different salts. I have avoided the use of the term 'membrane' in this connection, and have preferred to speak of the corpuscles as being bounded by an 'envelope,' a word which does not seem to carry with it the same preconceived histological and physico-chemical suggestions as the word 'membrane.' I have, however, demonstrated histologically (*Amer. Jour. of Phys., loc. cit.*) the existence of an envelope in the large nucleated corpuscles of Necturus. I hope soon to have an opportunity of returning to the subject.

of the laked blood by increasing the volume of the intercorpuscular liquid relatively to that of the corpuscles, and perhaps by increasing somewhat the degree of dissociation of the serum electrolytes, although it would diminish the specific conductivity of the intercorpuscular liquid.

4. An increase in the permeability of the corpuscles or ghosts to the ions, without, on balance, any increase in the electrolytes of the intercorpuscular liquid or in its amount, would increase the conductivity of the laked blood (apart from the depressing influence of the liberated haemoglobin).

According to the same writer, the difference in the action of the two groups of haemolytic agents may be explained in two ways :—

(*a*) By the assumption that in the first case a relatively small, and in the second case a relatively large amount of intracorpuscular electrolytes escapes from the corpuscles ; the relation of a considerable part of the electrolytes of the corpuscles to the stroma and envelope being such that an energetic action of the haemolytic on these structures is required for their liberation.

(*b*) By the assumption that the permeability of the envelope (and stroma) to the ions of the intercorpuscular liquid, and perhaps also to the intracorpuscular ions, is decidedly increased by the second group and slightly, if at all, by the first. The consequence of this would be that after the action of, say, a sufficient dose of saponin the laked blood would approximate more closely to the condition of a homogeneous conductor than after a less violent haemolysis, which left the shadows their 'semi-permeable' character.

Stewart arrived at the conclusion that an increase in the conductivity of blood after laking is not due altogether to a surrender of electrolytes by the corpuscles to the fluid, but rather that some alteration in the corpuscles which allows ions to pass through them more freely is caused by the laking process.

Foa, whose views as to the structure of corpuscles are quite at

variance with those of Rollett and of Stewart, observed[1] that repeated freezing and thawing of blood with nucleated corpuscles lowered its freezing-point by stages. He, believing that in the laking of non-nucleated corpuscles electrolytes do pass into the surrounding fluid, holds that any indications to the contrary furnished by measurements of the electrical conductivity of the fluid of laked blood are not admissible, in that we do not know what the relations between the albuminous components of the blood and the electrolytes may be, and what effect their relation may have on electrical conductivity. Moreover, by a method which he used, of separating the fluid of laked blood from the ghosts, he found the ash in the former in excess of that found in the serum of an equal quantity of unlaked blood.

Since it seems doubtful if a complete separation of serum and corpuscles, or of ghosts and fluid in laked blood, can be effected by simple centrifugalisation, I made, some time ago (at Dr. Stewart's suggestion), to test the constancy of the results, determinations of the ash in as much serum of measured quantities of blood and in as much intercorpuscular fluid of measured quantities of laked blood as could, after centrifuging, be separated as cleanly as possible from the corpuscular elements with a pipette. It was found impracticable—especially with laked blood, always darkened—to discern the exact line of separation; and there were always, even with the best available centrifugalisation, loose, flocculent particles, barely visible, above the seeming line of separation. This, I think, ought to preclude the possibility of an exact reading of the comparative volume of ghosts and of fluid in laked blood. It is less easy to imagine how a perfect separation of serum and corpuscles or fluid and ghosts can be effected by the method which Foa used of centrifuging in tubes with stop-cocks at the bottom, and then allowing the sediment to flow off through a small opening in the cock. If blood is centrifuged very long and rapidly, the corpuscles or ghosts will be packed together and pressed against the wall of the containing tube so tightly that they will adhere

1. *Archivio di Fisiologio*, Vol. I, Fasc. 2, 1904.

to the wall of the tube, and will continue to do so after the contents of the tube are poured out.

The numerous comparative determinations of the ash from serum and from the fluid of laked blood—made by trying to draw off all of the fluid parts carefully with a pipette—were made with measured amounts of chicken blood because it was expected that the corpuscles of such blood, still ballasted, so to speak, by the nuclei, would sediment well. The results are so inconstant that it is held to be wholly impossible to ascertain the ash from the fluid part of blood—especially when laked—by separating all of the fluid from the corpuscular elements.

With horse blood, also, determinations of the ash from the serum and from the fluid of the blood, laked by freezing and thawing, were made, the fluids being separated as completely as possible from the corpuscular elements after centrifuging a long while. The results of determinations in the fluid of the laked blood were far apart. The proportion between the amount of ash (L) found in the fluid of the laked blood and the ash (S) in the serum were even much greater in each case than Foa found with horse blood by his method of separation. Thus in two experiments I obtained :—

$$L : S :: 1{\cdot}196 : 1{\cdot}00$$
$$L : S :: 1{\cdot}3558 : 1{\cdot}00$$

which compares with Foa's result — $L : S :: 1{\cdot}1388 : 1{\cdot}00$

The gross irregularities in the increases in the ash of the fluid of the same blood laked by the same procedure but separated from the ghosts by such doubtful means, make it quite a certainty that some of these increases are due to salts which get into the ash determination in ghosts or in solid particles which do not sediment perfectly.

It seemed to me that it might be ascertained if salts come out of the corpuscles into the fluid on laking, by simply comparing the ash of a measured volume of serum of unlaked blood with a measured volume of fluid of laked blood, both being taken from the upper layers

only of portions well centrifuged in closed tubes, while at the same time the relative proportion of serum and corpuscles in the unlaked blood and of liquid and ghosts in the laked blood was determined by the haematocrite. But the same difficulty of effecting a complete separation of the ghosts presented itself here. I was, therefore, forced to leave out of account the possibility that the relative volumes of fluid and corpuscular elements of blood may change on laking without alteration in the proportion of salts. This would be the case if a quantity of water holding the same amount of salt as the same volume of serum, were transferred one way or the other. As, however, I found positive differences in the amount of ash, this objection does not render my observations valueless.

In these experiments the blood to be tested was handled in centrifuge tubes fitted with rubber caps, such as bacteriologists use. With these caps put on the tubes as soon as filled, there can be no evaporation and hence no alteration of volume of the contents. Some of the tubes were centrifuged, immediately after filling, to provide the normal serum; others were subjected to the laking process, well stirred up and then centrifuged. Carefully measured volumes of serum and of fluid of laked blood were pipetted off and brought into crucibles in which their ash-content was weighed after incinerating *lege artis*. In this way the relation of ash (S) in the serum of blood to the ash (L) in the fluid of the same blood laked was found in the following cases to be :—

L : S :: 0·917 : 1·00 (chicken blood laked by heat).
L : S :: 0·891 : 1·00 („ „ saponin).
L : S :: 1·0284 : 1·00 (horse blood laked by heat).
L : S :: 1·0544 : 1·00 („ „ saponin).
L : S :: 1·0618 : 1·00 (horse blood laked by freezing and thawing).
L : S :: 1·048 : 1·00 („ „ „ „).

In all cases where saponin was used as a laking agent the small amount of ash contained in it was deducted from the total ash of the incinerated laked fluid. In all cases where the ash contained iron derived from the haemoglobin of the fluid of laked blood it was included, since it was seen that the whole excess of L over S in the mammalian blood could be accounted for by the iron, and that was all I aimed at determining.

It will be seen from the above that the increase of ash from the fluid of horse blood laked by freezing and thawing is not nearly so large as Foa found by his method, and may be accounted for by the iron of the haemoglobin. If the intercorpuscular fluid contained only ten per cent. of haemoglobin (and it would almost certainly be more, since the whole, or nearly the whole, of the pigment is in the fluid), this would correspond, say, to 0·035 per cent. of Fe or 0·05 per cent. of Fe_2O_3. When the original serum contains 0·8 per cent. of ash this would make the ratio L : S :: 1·06 : 1, if no salts were exchanged between the corpuscles and the liquid. With chicken blood the quantitative relations above recorded between the ash of the serum and the ash from the fluid of laked blood are in accord with Foa's belief that there is a difference in the mode of laking of nucleated and the mode of laking of non-nucleated corpuscles. Stewart also found that in heat-laking of blood with nucleated corpuscles the conductivity might be markedly diminished.

On the other hand the relations found between the amount of the ash from the serum of blood with non-nucleated corpuscles and from the fluid of such blood laked in different ways are not easy to interpret. In blood laked by freezing and thawing we find that the increase of proportion of the ash from the fluid is almost as much in one series, and more in the other series than the increase of proportion of ash from the fluid of blood laked by saponin, whereas in blood laked by heat it is distinctly less.

Yet, as has been said, saponin increases the conductivity of the laked blood much more than heat or freezing and thawing. So far as these determinations go, then, they support the view that in none of these forms of laking is there a notable increase in the percentage of electrolytes in the intercorpuscular liquid, and that the increase in conductivity produced by saponin must be due either to an increase in the total volume of the liquid or to an increased conductivity of the corpuscles, or to both.

That a change in the conductivity of the corpuscles, not necessarily associated with the liberation of electrolytes, is caused by

saponin is indicated by determinations of ash in the fluid of suspensions of formaldehyde-hardened corpuscles treated with saponin, which bear out Stewart's view as to the behaviour of the corpuscles during the laking process. With suspensions of such corpuscles complete separation of the fluid can be made after centrifugalisation since they sediment to form a viscid mass from which the supernatant fluid can even be poured off; and the corpuscles can be washed and re-washed in M/4 cane sugar solution. Although formaldehyde-hardened corpuscles will not give up haemoglobin when subjected to the influence of laking agents, a suspension of them in isotonic solutions of salt will undergo an increase in its electrical conductivity when treated with saponin as great as if the corpuscles were fresh.

It is very easy to determine that the increase in conductivity of the suspension of formaldehyde-hardened corpuscles after treatment with saponin is not due to a passage of electrolytes from the corpuscles into the fluid of the suspension. Such suspensions, having been treated with weighed amounts of dried saponin containing a known amount of ash and allowed to stand from five to forty-eight hours, were centrifuged, and measured volumes of the supernatant fluid then taken for determinations of the ash obtainable from them, and these were compared with determinations of the ash from the supernatant fluid of a like centrifuged suspension, which had not been treated with saponin. It was found that the excess of ash from the supernatant fluid of the saponin-treated suspension could be accounted for by that added with the saponin when the suspension was in salt solution.

Or in other series, the suspensions of formaldehyde-hardened corpuscles in salt solution were first washed with M/4 solution of cane sugar and suspended in the latter when treated with saponin. After centrifuging such sugar solution suspensions, the ash from the supernatant fluid of the saponin-treated suspension was found to exceed that from the supernatant fluid of untreated suspensions in the exact proportions in which it was added with the saponin; or in a case where the whole supernatant fluid was taken for ash determinations, the excess of ash from the fluid of the saponin-treated

suspension was found to coincide almost exactly with that added with the saponin. The supposition that formaldehyde-hardened corpuscles on being treated with saponin, which raises the conductivity of a suspension containing them, do not give up any of their electrolytes to the surrounding fluid is therefore, by this method, well substantiated. The increase of conductivity must, then, be due to a change in the envelope (or stroma) which permits an easier passage to the ions of the liquid in which the corpuscles are suspended.

ON THE ACTION OF CERTAIN OXIDISING AGENTS UPON BLOOD-PIGMENT[1]

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While the destructive effects of chlorates on red blood corpuscles and the conversion of the haemoglobin into methaemoglobin have long been known the further effects have not been much studied. Formation of haematin, ultimate disappearance of absorption bands, and conversion of the blood into a dark solid mass have been stated to occur.[2]

Jellying of Blood and Colour Changes

The jellying effect of chlorate on blood is a remarkable one, and occurs as one of the results of the chlorate influence being prolonged much beyond the phase of methaemoglobin formation. It can be easily produced by mixing blood with crystals or strong solutions of sodium (*e.g.*, 10 per cent.) potassium or ammonium chlorate in amounts sufficient to give a strength of 2 to 5 per cent. chlorate in the mixture. When crystals are used the fluid must, of course, be stirred until solution is complete; otherwise there may be excessive percentages in certain portions of the fluid—with disturbing effects. With blood that has not been much diluted the jelly formed is very firm, coherent and elastic, and very difficult to reduce to a state of fine subdivision. Its formation is much accelerated by acidulation of the blood and by warmth (30° to 40° C.). When no acidulation is used the jelly

1. The main facts in this paper were communicated to the Physiological Society at the Oxford meeting in July, 1904, when illustrative specimens were shewn. Publication was delayed in the hope of carrying the investigation further in certain directions.

2. A list of papers is given at the end of the article on Chlorates in Cushny's Textbook of Pharmacology. See especially von Mering's paper in the *Berlin klin. Wochenschr.*, *No.* 44, 686 (1883).

is at first always dark red in colour when seen in mass—probably in twenty-four to forty-eight hours after the chlorate has been added, at room temperature. At a later stage, much hastened by acidulation and warmth, the red colour gives place to a deep green, as seen by reflected light, though thin slices viewed by transmitted light show a brownish-red tint. At a still later stage under the influence of decided acidulation and warmth the green colour may gradually change to a yellowish tint.

Sometimes the jelly contracts to a certain extent and squeezes out a clear fluid or serum. This fluid is rich in protein, etc., but gives no appreciable iron reaction with ammonium sulphide, ferrocyanide, etc., even after prolonged application of various methods for revealing 'masked' iron; the iron of the haemoglobin has evidently been retained in the jelly or clot. The squeezing out of fluid is very variable as regards its occurrence and amount; very often there is none.

When blood is diluted beyond a certain point jellying under the influence of chlorate fails and is replaced by a coloured precipitate varying in coarseness and tendency to coherence. The results are essentially similar whether the blood has been laked by dilution with water, etc., or diluted with isotonic salt solution. Ox blood which had been kept for many years behaved like fresh blood, and the results got with the blood of different animals (ox, horse, sheep, rabbit, rat, etc.), and with human blood, were essentially similar.

The influence of the reaction of the blood on these changes is very marked. Addition of alkaline salts (sodium carbonate, etc.) retards chlorate action very markedly, while acidulation accelerates the changes very strikingly. Samples of blood which were unusually alkaline shewed considerable resistance to the chlorate influence; the effect of slight acidulation of portions of the same blood was very notable. Hence the plan of administering alkalies in cases of chlorate poisoning has obviously a sound basis. Acidulation may be done by adding dilute acids or acid salts like acid sodium phosphate. With different samples of ox blood I have often used half to three volumes of 0·2 per cent. HCl, or about one volume of 0·5 per cent.

HNO_3. To make the mixture faintly acid to litmus paper varying amounts of acid are required, care being taken to guard against the acidity being sufficient to cause the formation of acid haematin, etc.; this was checked by the use of the spectroscope. Amounts of acid insufficient to make the mixture react acid to litmus are able to favour chlorate action markedly.

The relations of the development of the colour changes to the jellying vary considerably. With acidulation and warmth the green phase may be arrived at very early, indeed almost as soon as the jellying, while with an alkaline reaction in the cool the jelly may remain red for many days. Similarly, when the blood is diluted so much that a flocculent precipitate occurs instead of jelly formation the particles may become green at a very early stage in presence of pronounced acidulation.

Under certain conditions the blood, especially when diluted to a certain extent, may, after the addition of chlorate, present the appearance of a deep green fluid with little or no jelly or precipitate. Such conditions are (1) when the reaction is too alkaline or insufficiently acid, (2) when the percentage of chlorate is insufficient, and (3) when the temperature is too low, the reaction and amount of chlorate present being such as to require warmth for complete precipitation. The addition of acid (*e.g.*, acetic) in such cases usually leads to precipitation or jellying immediately or in a short time; if much acid is added, the development of the yellow phase is brought on. Alcohol also causes precipitation in the green fluid, the coloured substance being thrown down among others.

The effects obtained with bromates were quite similar to those following the use of chlorates. Iodates on the other hand, have not the same action; a red precipitate is thrown down with properties differing from those got with chlorates and bromates, the blood not being acidulated.

Similar results are obtained when washed red blood corpuscles are used instead of blood.

Solutions of the stromata were prepared by Halliburton's[1]

1. *Journ. of Physiology*, Vol. X, p. 532.

modification of Wooldridge's method, and these were tried with chlorate in the same way as blood, but no jellying or precipitation followed. On the other hand the solution of haemoglobin left after removal of the stromata gave the characteristic results with chlorate—very rapidly indeed on account of the previous acidulation with acid sodium sulphate used to precipitate the stromata.

Blood plasma and serum were found to give negative effects with chlorate, whether acidulated or not; acid sodium phosphate was used to acidulate. Even when kept in a warm chamber for considerable periods this was the case; except where the plasma or serum had been contaminated with some haemoglobin—leading to precipitation by the chlorates—the serum pigment remained apparently unchanged, at least for long periods. Similarly negative results were obtained with egg-albumin.

Effects on Solutions of Haemoglobin

From the foregoing observations it is evident that striking effects of chlorates on the blood are essentially effects upon the haemoglobin, and not upon the ordinary proteins. This conclusion was confirmed by the characteristic effects got with solutions of haemoglobin crystals and of methaemoglobin; the former were prepared by different methods, commonly by some of the modifications of Hoppe-Seyler's method. The same phenomena as regards colour changes, jellying, etc., were readily observed. Samples of haemoglobin obtained from Grübler, Merck and others were also used. Solutions of CO-haemoglobin were found to behave in much the same way. As a rule no acidulation was found to be necessary with the haemoglobin solutions; the chlorate or bromate was simply added to the usual amount. Cautious acidulation accelerates the changes.

Haematin solutions on the other hand quite fail to give these results; if blood is treated in such a way as to break up its haemoglobin into haematin and globin, treatment with chlorate in the usual way does not produce the effects seen when unchanged blood or haemoglobin is dealt with.

Separation and Properties of the Modified Haemoglobin

From haemoglobin solutions as well as from blood the product was separated in conditions of greater or less purity. When blood was used the most successful results were obtained when considerable dilution was employed, *e.g.*, ox blood diluted with sixteen volumes of water and cautiously acidulated with 0·2 per cent. HCl, or 0·1 to 0·5 per cent. HNO_3, chlorate or bromate being then added to the amount of 2 per cent. Methaemoglobin was rapidly formed and soon a flocculent precipitate appeared—very marked in less than half an hour. Centrifugalised and subjected to repeated and thorough washing first with normal saline and then with distilled water. Boiling was tried with one portion, and helped the separation of the precipitated matter without causing any evident change of colour.

In other cases the blood was diluted only three or four times with very weak acid and after addition of chlorate or bromate kept constantly stirred to prevent jellying, a coarse flaky precipitate (green) being got in an hour or two; this was washed thoroughly.

The substance obtained in the dry state is a dark powder (looking somewhat like haematin) with metallic lustre.

Microscopically it presents the appearance of yellow or brownish-yellow polygonal plates, varying much in size and form. With the micro-spectroscope no absorption bands are visible. Slices of the coloured jelly in the moist state shew similarly negative appearances with the spectroscope. The methaemoglobin spectrum resulting from the addition of chlorate to the blood gradually disappears as a result of more intense and prolonged influence.

The dried substance is very stable and insoluble in water and most reagents, *e.g.*, alcohol, ether, chloroform, benzol, glycerine, etc. Also in acids like HCl and H_2SO_4, in solutions of alkaline salts and in weak solutions of caustic alkalies, while it dissolves after a time in strong solutions of NaOH (20 per cent., etc.) and KOH. A certain amount of solution takes place after many hours in weak alkalies in the warm chamber. The freshly precipitated moist substance is more soluble in dilute alkalies. Boiling causes no obvious effect upon the substance;

its colour—whether red or green—remains unchanged. When a solution has been made with strong alkali, boiling with lead acetate gives a negative result as regards reduction.

In 0·2 per cent. HCl the solution swells up but does not dissolve even with the aid of warmth.

A solution in strong alkali shows no definite absorption bands, and when treated with ammonium sulphide shows only a very faint haemochromogen spectrum.

Treatment of the substance with H_2SO_4 does not give anything resembling haematoporphyrin, or indeed any definite bands.

Question of Formation of Halogen Compound

In view of the results that have been obtained by Mulder[1] and various more recent workers on the combination of proteins with halogens, and especially those described by Hopkins and Pinkus[2] and by Kurajeff[3] (combination of haemoglobin with iodine), the question suggests itself as to whether there is any combination of such a kind here. Examination of the chlorate and bromate products by Carius' method does not lend countenance to this suggestion, the halogen found being apparently of the nature of an impurity.

The red, green and yellow products obtained by the action of chlorate and bromate seem to be oxidation products of haemoglobin.

Retention and Liberation of the Iron

The substance retains all the iron of the haemoglobin from which it was formed, and the iron is in very firm combination. There is no reaction with ferrocyanide and HCl, with Macallum's[4] haematoxylin solution, nor with ammonium sulphide even when kept for long periods in the warm chamber. This is the case if the chlorate action in the preparation of the substance from haemoglobin solution or blood is not carried too far, in the presence of too much acid, etc.

1. *Journ. f. pr. Chem.*, Vol. XLIV, s. 487.
2. *Ber. d. deutsch chem. Gesellsch.*, Vol. XXXI, s. 1311 (1898).
3. *Zeitsch. f. physiol. Chemie*, Vol. XXXI, s. 527 (1901).
4. *Journ. of Physiology*, Vol. XXII, p. 25.

The presence of Fe in the solution can be demonstrated in the usual way by incineration, etc., or by setting free the iron by prolonged treatment with acid alcohol (Bunge's fluid, etc.) and warmth, or more easily by chlorate or bromate plus *strong* acidification and warmth. By using 10 per cent. solution of sodium chlorate with $\frac{1}{10}$ to $\frac{1}{5}$ volume of saturated salicyl-sulphonic acid in the warm chamber the whole of the Fe is usually removed in a day or two; as soon as it is set free the ferric salt strikes a red colour with the salicyl-sulphonic acid and the progress of the extraction is readily gauged by the depth of the coloration. When the Fe has been completely removed in solution an orange-yellow residue is left.

A similar method serves for the extraction of Fe from ordinary haemoglobin or from blood. A weaker solution suffices to extract a good deal of the Fe even at room temperature, and with such a solution (*e.g.*, containing 5 per cent. salicyl-sulphonic acid) testing with ferrocyanide can be readily done (without HCl), and with ammonium sulphide after neutralisation.

The iron of haematin may also be extracted by treatment with chlorate whether treated as (1) haematin in the solid state, HCl, etc., being used for strong acidulation, or (2) haematin dissolved in glacial acetic acid or salicyl-sulphonic acid alcohol. Heat accelerates the extraction. When the Fe is completely removed a yellow substance is left which is not ordinary haemato-porphyrin.

As regards the possibility of demonstrating the iron *in situ* in the modified haemoglobin or in ordinary haemoglobin by means of ammonium sulphide, ferrocyanide, etc., the difficulty is that when the Fe is set free it is readily dissolved out. In order to prevent this I sought a solution which should liberate the combined Fe but not dissolve it out, and obtained such by dissolving salicyl-sulphonic acid in ether to saturation. Treatment with this fluid liberated the Fe but did not remove it, and it was possible to stain it *in situ* in the crystals and in blood corpuscles. But in the latter case the injurious effects of prolonged action of the fluid constitutes a serious drawback as regards histological applications. In the case of the modified haemoglobin got by means of chlorate or bromate the substance is

so resistant that it was found quite possible to obtain successful preparations shewing the iron *in situ* by the black coloration with ammonium sulphide and with haematoxylin, and the blue with ferrocyanide.

Effects of Peptic and Tryptic Digestion

Digestion with peptic fluid gave results strikingly different from those obtained with ordinary haemoglobin or methaemoglobin. Instead of there being a splitting of the pigment into the protein component and acid haematin which becomes precipitated, the substance in this case gradually dissolves forming a yellow solution in which proteoses and, later, peptone can be recognised. No haematin appears, and in the earlier stages there is little or none of the iron liberated; though with prolonged digestion an extensive setting free of iron occurs and some yellowish deposit may form in the fluid. The rapidity of digestion varies much with the exact condition of the substance tested; in the moist state and well subdivided (*e.g.*, a recently-formed flocculent precipitate) it is very rapidly dissolved (two to three minutes, etc.); the dried product on the other hand is much more slowly acted upon. No absorption bands are visible in the yellow solution. The digestibility of the substance is very notable in view of its generally insoluble and resistant character. Whether this material can be turned to useful account as a source of 'organic' iron for therapeutic or nutritive purposes remains to be seen.

When a neutralised digest taken at an early stage is dialysed it may be found that a certain amount of combined iron passes through into the dialysate and can be demonstrated there by methods for revealing the presence of 'masked' or 'organic' iron, *i.e.*, there is a certain amount of iron combined in the form of a diffusible compound. But if peptic digestion is at all prolonged the iron gradually becomes liberated. If treatment with the chlorate has been carried on too long, or too decided acidulation has been used, iron seems to be set free at an earlier stage of digestion.

Pancreatic fluid is similarly effective in digesting the substance

in question; alkaline haematin is not formed as in the digestion of ordinary haemoglobin. With tryptic digestion the liberation of iron seems to be slower than with peptic fluid.

Treatment of blood or haemoglobin with a soluble persulphate (*e.g.* 2 to 3 per cent. sodium persulphate) also gives a modification of haemoglobin which is completely digested by peptic and pancreatic fluids without splitting off haematin. In the case of blood the modified haemoglobin is mixed with other blood proteins also coagulated by the reagent.

Some Applications of the Chlorate and Bromate Action upon Haemoglobin

In virtue of the above described action in coagulating haemoglobin while not similarly affecting ordinary proteins, chlorates and bromates may in certain cases be successfully used to separate haemoglobin from other proteins, etc., a suitable degree of slight acidification being provided by the use of acid sodium phosphate, etc., as in the case of serum, etc. The same may be done in urine containing haemoglobin (using 5 per cent. of the chlorate at room temperature for a day or two) with proper acidulation, but here certain constituents often interfere with the separation of the haemoglobin. The chlorate causes no precipitation in normal urine.

When it is desired to liberate and demonstrate the iron of the haemoglobin, *e.g.*, by the use of chlorate and strong acidulation with salicyl-sulphonic acid as already described, complications arise from the fact that certain urinary constituents (uric acid, kreatinin, etc.) discharge the red colour struck by the liberated iron with the salicyl-sulphonic acid—unless the amount of iron be large; ammonium sulphide and ferrocyanide may likewise fail to demonstrate the presence of appreciable amounts of iron. Interfering constituents are also present in muscle extract, but not to any marked extent in serum or plasma.

In the absence of such disturbing agents it is very easy to liberate and demonstrate the iron of haemoglobin, etc. For class purposes two or three drops of blood may be mixed with sodium chlorate

solution (5 to 10 per cent.) and acidulated with salicyl-sulphonic acid to the amount of 2 to 5 per cent. The tube is left to stand for twenty-four hours, and the liberated iron shews by pink coloration of the fluid, which can also be tested by ferrocyanide and (after neutralisation with ammonia) by ammonium sulphide.

The pigments of serum, bile and urine are not precipitated or decolourised by treatment with chlorate (after acidulation with acid sodium phosphate) over periods vastly longer than are necessary to change haemoglobin in the way described.

Chlorate and bromate may be used as fixing agents for haemoglobin for certain histological purposes, etc., *e.g.*, in the case of the natural injection of organs and parts with blood.

ON THE PRESENCE OF OXYDASES IN INDIA-RUBBER, WITH A THEORY IN REGARD TO THEIR FUNCTION IN THE LATEX

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In a previous paper[1] on the distribution of the protein in Para rubber, I have shown that Weber's insoluble oxygen-addition compound of india-rubber is in reality of the nature of vegetable protein and have attempted to account for the singular behaviour of this insoluble protein towards solvents by a physical theory based on its peculiar distribution throughout the rubber in the form of a fibrous network.

In this same paper I drew attention to the rather remarkable striped appearance which moderately dry sections of raw Para rubber exhibit, and as the peculiar shading of colour in the form of dark and light brown layers alternately throughout the rubber seemed to correspond with the distribution of the protein in the latter as evinced by the microscopical examination of stained sections, I suggested that the protein had probably a not unimportant function in the raw product.

These incidental observations in regard to the properties of the insoluble constituent of Para rubber led me, however, to investigate still further the nature of the nitrogenous products which I have since found to be present not only in Para rubber, but in greater or less amount in some forty different brands of raw rubber obtained from the various rubber-producing plants throughout the world.[2]

1. *Quart. Journ. Liverpool Institute of Commercial Research*, Vol. III, No. 6, p. 47, 1908.

2. There seems indeed to be little doubt that nitrogenous products of a similar character are to be found in greater or less amount in the latex of all the rubber-producing trees or vines. Furthermore in the processes for the coagulation of the latex, unless special steps be taken, the protein invariably becomes coagulated and bound up to a great extent in the rubber clot, giving rise to putrefaction and deterioration in badly-cured samples.

Some time ago my attention was directed to a short note by Tschirch and Stevens[1] on oxidizing enzymes in gums. The singular resemblance in solubility and in general behaviour of these enzymes to the insoluble constituent of Para rubber led me at once to examine this rubber for oxidizing enzymes, and to attempt to determine in how far the rapid darkening in colour and the oxidation of raw rubber in general might not be accounted for by the presence of such an enzyme in the insoluble constituent of india-rubber.

Before proceeding, however, to describe the experimental work which has led to the isolation and identification of such an enzyme, let me briefly outline the literature on this subject.

As the result of numerous investigations in the field of plant physiology, our knowledge of the nature of oxidizing ferments and of their function in the plant has been considerably increased within the last few years, and it must be looked upon as a great step in advance that experimentation, in particular the work of Bach and Chodat, has shown us that it is necessary to distinguish sharply between the 'direct oxydases' and the peroxydases (indirect oxydases) which act only in presence of hydrogen peroxide or another peroxide body.[2]

Nor can the function of these enzymes in the vital oxidation processes be overlooked, since many oxidation phenomena have been shewn to be due to the intermediate formation of peroxides and the action of oxidizing enzymes, while other important oxidation processes taking place in the plant have been attributed to the action of specific oxidizing enzymes.

The colour changes observed in freshly-cut vegetable tissues exposed to the air have been generally ascribed to the action of oxydases, in many cases without even experimental proof of the existence

1. Separatabdruck aus der *Schweiz. Wochenschrift für Chemie u. Pharmacie*, No. 31, 1905.

2. A review of the present day theories in regard to the nature and the function of oxidizing enzymes in plants is given by Bach and Chodat (*Biochem. Zentralblt.*, Vol. I, p. 416, 1903); a very complete account of all the literature on this subject is to be found in Czapek, '*Biochemie der Pflanzen*,' Vol. II, pp. 464-481, 1905. According to Bach and Chodat the oxydases are a mixture of two enzymes of distinct types; the oxygenases or protein-like bodies, exceedingly unstable towards heat, chemical agents, etc., which take up molecular oxygen from the air with peroxide formation; the peroxydases or enzymes of nitrogenous but non-protein character which are much more stable towards heat, etc., than the oxygenases and activate the oxygenase, causing it to give up oxygen to the auto-oxidisable substrate. That the oxygenase fails in the extracts of some plants is due to its great instability.

of such enzymes in the tissues in question. It is not, therefore, surprising that the gradual darkening in colour which has long been observed to take place in freshly prepared samples of raw rubber, became associated in the minds of observers at a very early stage with the presence of an oxydase in the raw product. Czapek,[1] indeed, states that the latex of rubber-producing plants contains an oxydase which is partially responsible for the darkening in colour of the coagulated product. Weber[2] attributed the intense darkening in colour of Castilla latex to the presence of an oxydase, without, however, investigating this point, while Tschirch[3] states that the darkening in colour of raw rubber is due to the action of enzymes (oxydases). I have not, however, been able to find any reference to experimental evidence supporting these views, and on the other hand Schidrowitz and Kaye[4] have reported that they examined a sample of Hevea latex but failed to get any indication of the presence of oxydases. The latex, however, which they examined could not be regarded as a normal one seeing that it contained barely 4 per cent. of dry rubber. That oxydases occur in the latex tubes of certain plants Raciborski[5] has shewn, and the presence of a similar enzyme (laccase) in the gum of Japanese lacqueur has been shewn by Bertrand[6] and others. Furthermore, the presence of oxydases in gum arabic and indeed in almost all gums has been determined by Struve,[7] Bertrand and Bourquelot,[8] Wiesner,[9] Tschirch and Stevens,[10] and important biological functions have been ascribed to these enzymes by certain authorities.[11]

Numerous reagents have been suggested and employed successfully

1. Czapek, '*Biochemie der Pflanzen*,' Vol. II, p. 705, 1905.
2. Weber, *Ber. chem. Ges.*, Vol. XXXVI, p. 3110, 1903.
3. Tschirch, '*Die Harze und die Harzbehälter*,' 2nd edit., Vol. I, p. 992, 1906.
4. *India Rubber Journal*, Vol. XXXIV, No. 1, p. 24, 1907.
5. *Ber. d. deutsch. bot. Ges.*, p. 52, 1908.
6. *Compt. rend.*, Vol. CXVIII, p. 1215, 1894; Vol. CXX, p. 266, 1895; Vol. CXXI, p. 166, 1895; Vol. CXXII, p. 1132, 1896.
7. *Lieb. Ann.*, Vol. CLXIII, p. 160.
8. *Compt. rend. soc. biol.*, Vol. XLIX, 1897.
9. *Sitzb. d. Wien. Akad.*, 1885, and *Monatsb. d. Chem.*, Vol VI, p. 592, 1885.
10. *Archiv. d. Pharm.*, p. 535, 1905, and *Pharm. Zentralb.*, Nr. 26, 1905.
11. See Tschirch, '*Die Harze und die Harzbehälter*,' 2nd edit., Vol. I, p. 883, 1907.

from time to time for the detection of oxydases, and it has been shewn that the behaviour of these enzymes towards reagents may vary with the source of the oxydases (certain oxydases giving a negative reaction to one class of indicator and a positive reaction with another). One cannot, therefore, rely on the tincture of guaiacum reaction alone in studying the action of oxydases. For this reason, in the work of isolating the peroxydase from Para rubber which I now propose to describe, I have been led to study the behaviour of the enzyme towards some ten well known reagents for oxydases which will be described in due course, and have only used the reaction with guaiacum as a rough and ready test in preliminary experiments. Further, my original intention in commencing this work being to clear up certain points in regard to the so-called insoluble constituent of Para rubber which has been surrounded by mystery in the past, my attention has been confined in the following communication to Para rubber only.[1]

Experimental

Preliminary experiments to isolate an oxydase from raw Para rubber were carried out as follows :—

A large quantity of unwashed raw Para rubber, cut from the interior layers of a very large block, was cut up into very fine sections by means of a sharp knife. These sections, which were as thin as it was possible to cut them, were allowed to soak in a quantity of distilled water sufficient to cover them, for a period of seven to ten days, after which time the aqueous extract was filtered off. About 100 grammes of the finely minced rubber were treated in this way each time, and the extract was tested from time to time by means of tincture of guaiacum with and without hydrogen peroxide. The first experiment showed that it was possible in this way to extract a ferment which had the property of rapidly turning tincture of guaiacum in presence of hydrogen peroxide a fine deep blue, and which was destroyed by boiling (peroxydase). The watery extract was alkaline to litmus, and appeared to give marked indications of the presence of protein by the Millon's and other tests. In order, therefore, to purify the extracts as far as possible they were dialysed for twenty-four hours against running water, preliminary experiments having shewn that in this way the peroxydase activity towards guaiacum was not destroyed.

1. It is hardly to be doubted, however, that what has been shewn to hold for the insoluble nitrogenous body in Para rubber, will also be shewn in time to be equally true for the insoluble nitrogenous product to be found in all varieties of raw india-rubber. Tschirch's work on the insoluble nitrogenous body in the various gums, and the gradual darkening in colour of all the ordinary varieties of india-rubber on the market only serve to support this view.

The dialysed aqueous extract was found to have all the properties of a powerful peroxydase (indirect oxydase) but to be practically devoid of activity in absence of hydrogen peroxide. It was tested as follows :—

Three test tubes were taken and into each was pipetted 10 c.c. of the dialysed extract. To serve as a control one of these was then heated to boiling for a few minutes and cooled, after which a measured quantity (1 c.c.) of a dilute standard solution of hydrogen peroxide was added to it and also to one of the other two quantities of active extract. The three tubes were then set side by side while the same quantity of a standard solution of the indicator to be used was added to each.[1] The activity of the dialysed extract was tested in this manner with reference to the following reagents :—Tinct. guaiacum, p-phenylenediamine, o-phenylenediamine, *a*-naphtol, phenol-phthalin, hydrochinon, amidol, pyrogallol, p-phenylenediamine + *a*-naphtol (indophenol reaction), tyrosin. The results observed are given in the following table :—

Reagent	Ten c.c. of the extract boiled, and hydrogen peroxide added	Ten c.c. of the extract without hydrogen peroxide	Ten c.c. of the extract with hydrogen peroxide added
Tinct. guaiacum[2] ...	Pure white ...	Pure white, unchanged	Deep blue in five mins.
p-phenylene-diamine[3]	Colourless ...	Colourless ...	Dark brownish red
o-phenylene-diamine[3]	Faint yellow ...	Colourless ...	Yellow, finally red
a-naphtol[4]	Colourless ...	Colourless, after 2 hours	Marked brown
Phenol-phthalin[5] ...	Faint pink ...	,,	Deep pink
Hydrochinon[6] ...	Colourless ...	,,	Intense port red
Amidol[7]	Faint pink ...	Faint pink ...	Deep cherry red
Pyrogallol[8]	No change after 24 hours	No change ...	Yellow coloration and brown deposit
'Indophenol' Mixt.[9]	Faint colour ...	No change ...	Deep violet
Tyrosin[10]	No action ...	No action ...	No action

1. It is hardly necessary to point out that the solutions of these reagents were prepared fresh immediately before use. Peroxide formation in the process of auto-oxidation of these reagents may lead to erroneous results. See '*Die Peroxydasereaktion der Kuhmilch, etc.*,' Dr. P. Waentig, *Arbeiten aus dem kaiserlichen Gesundheitsamte*, Vol. XXVI, No. 3, 1907.

2. Van den Broek, *Jahresber. Chem.*, p. 455, 1849-50; and Schonbein, *Zeit. f. Biol.*, Vol. IV, p. 367, 1868. An alcoholic extract of the powdered resin.

3. Aqueous solutions of the hydrochlorides of the bases.

4. Solution in 50 % alcohol containing trace of alkali.

5. Kastle and Shedd, *Amer. Chem. Jour.*, Vol. XXVI, p. 527, 1901.

6. Dilute solution in water.

7. The developer Amidol has not been previously used. As a rapid indicator of oxydase activity it was found to be very useful, giving an intense dark red coloration within a few minutes. An aqueous solution acidified with a few drops of 5 % acetic acid was used.

8. A solution in water. The oxidation proceeds slowly, an insoluble brown precipitate of purpurogallin separating out in the course of 24 hours in the presence of oxydase.

9. Rohmann and Spitzer, *Ber. chem. Ges.*, Vol. XXVIII, p. 567, 1895. The reagent contains 1 mol. *a*-naphtol, 1 mol. p.-phenylenediamine, and 3 mol. Na_2CO_3 in water.

10. A dilute solution in water containing a trace of Na_2CO_3.

These tests suffice, I think, to shew beyond all doubt that the dialysed aqueous extract from Para rubber contains a peroxydase enzyme or enzymes of the general type, readily destroyed by boiling. That the extract had no action on tyrosin points to the absence of a tyrosinase. Extracts from several different samples of Para rubber were examined in this way under varied conditions and always with the same result. The extract had also the property of a catalase, 20 c.c. of this extract in one experiment in an eudiometer tube over mercury liberating 12 c.c. of oxygen from hydrogen peroxide within a few hours; there was also a very slight indication of proteolytic activity.[1]

The dialysed extract was neutral to litmus, and gave neither the xanthoproteic reaction or the familiar coloration for protein on warming with Millon's reagent. No definite biuret test could be got with the dialysed extract. Boiled with a trace of acetic acid the extract became opalescent, and a small quantity of coagulated matter separated.

Very small quantities of acid or alkali did not appear to influence the peroxydase activity to any extent although large quantities of either of these reagents stopped its action completely.

When the dialysed extract was concentrated in vacuo over sulphuric acid it appeared to lose in activity gradually and became finally inert, the sulphuric acid colouring at the same time due to absorption of ammoniacal products. This method, then, obviously could not be used for the isolation of the peroxydase, nor was the method of saturating the extract with ammonium sulphate of much value in this connection.

For the isolation and purification of the peroxydase the following method was found to be most satisfactory:—

The finely cut raw Para rubber was digested for about a week with 40 per cent. alcohol, after which the alcoholic extract, which became intensely yellow during the process due to the extraction of resins, etc., from the rubber, was filtered off and precipitated with several times its own volume of absolute alcohol. On standing, a gummy mass settled out which was filtered off, redissolved in 40 per cent. alcohol, and again precipitated with absolute alcohol. When this process was repeated three times a product was obtained which was infinitely more active than the original precipitate, blueing tincture of guaiacum in presence of peroxide practically instantaneously.

1. Metts digestion tubes.

Properties of the Peroxydase from Para Rubber

The product obtained in this way no longer appeared to possess either catalytic or proteolytic activity to any extent, and in the moist state was a gummy looking mass which left a dry vitreous solid on evaporation of the alcohol. The amount of this solid extracted from the rubber was very small, so that a kilogramme of Para rubber had to be extracted in order to obtain sufficient material for chemical tests.

The peroxydase dissolved slowly in water, and the solution was found to be many times more active in presence of peroxide than the original aqueous extracts,[1] although it had not the slightest action in absence of hydrogen peroxide.

The specific activity of the aqueous extract of the peroxydase was destroyed by the addition of small quantities of potassium cyanide, sodium fluoride, or mercuric chloride to it, while in presence of mineral acids and alkalies, the activity also disappeared. The activity of the peroxydase was found to diminish gradually when the aqueous solution was left exposed to the air and light for some time.[2] Towards heat the peroxydase was remarkably stable, and it is interesting to note in this connection that when its solution in water was just raised to the boiling point, and then allowed to stand for some time, its activity gradually returned to a slight extent so that it again gave a marked reaction with tincture of guaiacum or with the indophenol reagent.[3]

By a series of rough measurements with tincture of guaiacum colorimetrically and with pyrogallol, the activity of the peroxydase was determined after exposure to different temperatures. It was found that heating the aqueous extract for 5 minutes to 80° C.

1. It is unnecessary to repeat here the various tests which were carried out in order to determine the peroxydase nature of the substance in solution. All the reactions, however, which have been described on page 169, were repeated many times during the course of the work, and always with more or less the same result.

2. Bach (*Ber. d. chem. Ges.*, Vol. XLI, p. 225, 1908) has determined a reduction in the activity of peroxydase due to the action of oxygen and light.

3. These observations agree with those of Woods (*United States Dep. of Agr. Bull.*, No. 18, p. 17) who found that the oxydase from tobacco juice when killed by boiling, gradually regained its activity on standing. Both Woods and Aso (*Bull. Coll. Agric., Tokyo*, Vol. II, p. 220) appear to be agreed on the existence of a zymogen, more stable towards heat than the oxydase, which changes slowly into the active enzyme.

destroyed the peroxydase activity completely, while 2½ minutes reduced the activity by one half. Heated to 70° C. for 5 minutes the peroxydase was still active, whereas after 15 minutes the enzymic action was completely destroyed. The peroxydase when heated for 10, 20, 30 or 40 minutes to 60° C. was not destroyed.

The thermal optimum of the peroxydase was determined by similar colorimetric methods and it was found to be about 55° C.

In regard to the chemical nature of the peroxydase the following points were noted :—

Although the peroxydase contained a considerable amount of nitrogen, it gave no reaction either with Millon's reagent or by the biuret or other tests for protein. When fused with KOH, ammonia was evolved in quantity along with traces of pyrrol which gave the characteristic red coloration to a splint of pine-wood moistened with hydrochloric acid.[1] This point will be dealt with again in full when the properties of the oxygenase from the latex are discussed. The peroxydase, however, gave a slight reduction with Fehling's solution, and further gave the characteristic coloration for pentoses[2] with phloroglucin and hydrochloric acid, so that the pyrrol ring is probably formed from the pentose and ammonia set free in the process of fusion.

The peroxydase isolated from Para rubber was found to give a very marked reaction for iron. The coloration with potassium ferrocyanide or with ammonium sulphocyanide was only given, however, when the solution of the peroxydase was treated in such a way that the activity of the enzyme was destroyed. Thus for example, although no coloration was given by adding either of these reagents directly to the peroxydase solution when a drop of dilute hydrochloric acid was first added to the peroxydase or when the peroxydase

1. Tschirch was the first to observe that pyrrol is evolved when the oxydase from Japanese lacqueur is distilled with KOH (*Archiv. der Phar.*, 243, 7, 504, 1905. Bach has recently confirmed Tschirch's observations in connection with the peroxydase from the radish, but has pointed out that ammonia as well as pyrrol vapours are evolved by this reaction (*Ber. d. deutsch. chem. Ges.*, Vol. XLI, p. 226, 1908).

2. From a review of the literature on this particular point it appears that a carbohydrate group of a reducing nature is to be found associated with most of the oxidizing enzymes, and that it is extremely difficult if indeed not impossible to separate this complex from the oxydase. Whether in reality a compound between carbohydrate and enzyme exists still remains to be shewn. See Bach and Chodat, *Ber. chem. Ges.*, XXXVII, p. 42, 1904; Cazeneuve, *Compt. rend.*, Vol. CXXIV, pp. 406 and 781, 1897; Tschirch u. Stevens, *Archiv. der Pharm.*, 243, 7, 535, 1905.

solution was boiled for a few minutes (although less marked in this case) and then treated with these reagents, a fine pink coloration was got ~~with the~~ ammonium sulphocyanide and a deep blue precipitate with the ferrocyanide reagent. Without going into a discussion of the present day position in regard to the function of the manganese,[1] which is usually associated with the oxydases or of the iron[2] which has already been found in an oxidizing-enzyme and is probably present in a colloidal form therein, from the above observations I am inclined to believe that considerable importance must be attached to the iron-content of the peroxydase from Para rubber.

Relationship of the Peroxydase to the Insoluble Protein of the Rubber

Having thus determined the presence of a peroxydase enzyme, it became of interest to study more closely its relation to the so-called insoluble constituent or protein in the rubber. The peculiar striped appearance of Para rubber, which, as I have already pointed out, falls closely in line with the distribution of the protein as revealed by the microscopical examination of stained sections, gave a valuable indication of the oxydase nature of the latter.

Indeed, the presence of a peroxydase alone in the insoluble body would not appear to be sufficient to account for the rapid darkening in colour observed when freshly cut rubber is left exposed to the air. According to Chodat and Bach the peroxydase is inactive unless in presence of the peroxide forming enzyme (oxygenase) or preformed peroxide, and this at once led me to examine the insoluble constituent of Para rubber for an oxygenase-complement which had not been extracted in previous experiments. Here, however, almost negative results were obtained, and it was only later when fresh latex from *Funtumia elastica* was obtainable, that I was able to prove the presence of an oxygenase as well as of a peroxydase in the fresh latex (see page 175).

1. Bertrand, *Ann. chim. phys.* (7) Vol. XII, p. 115; *Compt. rend.*, CXXIV, pp. 1032, 1355 (1897); *Bull. soc. chim.* (3) Vol. XVII, pp. 619, 753 (1897).

2. Sarthou, *Journ. pharm. chim.* (6) Vol. XI, p. 583, 1900; Vol. XIII, p. 464, 1902.

The method by which the Para rubber was tested for the presence of an oxygenase was as follows :—

A quantity of the insoluble protein was isolated from Para rubber by dissolving away the rubber in chloroform[1] in the cold. After three months' extraction the insoluble product left was washed with alcohol and dried. It was a hard, stringy, but non-elastic mass which gave a slight reaction for protein with Millon's reagent.

This body was allowed to act on peroxydase extracts in test tubes, but when the extracts were tested they shewed not the slightest activity in absence of hydrogen peroxide. As the oxygenase, as is conceivable, might have been destroyed by the long continued action of chloroform, thin sections of the raw rubber were cut and these were placed directly in a peroxydase extract with tincture of guaiacum. Whereas the tube containing the rubber and extract was only very faintly blue in colour and was hard to distinguish from the control with extract alone, the extract in presence of hydrogen peroxide gave a deep blue colour in a very short time.

Numerous experiments on these lines were tried in order to determine the presence of an oxygenase along with the peroxydase in the insoluble constituent of Para rubber, but the insuperable difficulties connected with the isolation of the 'insoluble constituent' from Para rubber made all attempts in this direction useless. One is led to the conclusion, therefore, that either the oxygenase is killed in extraction experiments with chloroform and toluol, and is not directly got at in the other tests on account of the large mass of the colloid material with which it is associated, or else that the oxygenase fails entirely in the rubber, being destroyed in the coagulation of the Hevea latex.[2] In view of the activity of the peroxydase in the rubber as evinced by the colour-changes produced, the first explanation seems to me to be the more probable of the two. The subsequent examination of a sample of *Funtumia elastica* latex and the isolation of an oxydase therefrom, determines with a fair degree of certainty the existence of an oxydase in the raw rubber.

In conclusion, further support was given to the theory of a stable zymogen in the raw rubber, by the fact that boiling the rubber with water alone or with water containing small quantities of cyanide, mercuric chloride, or formic acid for some time did not prevent the further darkening in colour of the rubber on prolonged keeping.

1. See 'Distribution of the Protein in Para Rubber,' *Quart. Journ. Inst. Comm. Res.*, Vol. III, No. 6, p. 51, 1908.

2. It would be interesting in this connection to know approximately the temperature at which coagulation of the Hevea latex is brought about.

Oxydases in the Latex

It may seem out of place that the examination of the latex emulsion was deferred until this point. Obviously, if any reaction is to be found it ought to be sought for in the fresh latex from which the rubber is formed. But various difficulties have to be overcome in working with latex, there being the question of supply of an emulsion which is not to be had in this country, and is withal exceedingly unstable and liable to coagulate. Hence it is that what should in the ordinary course of events have been studied first had to be left until late in the research.

A quantity of latex from *Funtumia elastica*, Stapf., preserved according to my directions, arrived here recently from Southern Nigeria.[1] The latex, which had been carefully collected and sealed up in well-filled pint bottles, arrived in this country for the most part in perfect condition. As only a couple of the samples of this latex come under consideration here, these only will be dealt with. The others will be considered elsewhere.

The two samples submitted to thorough examination for oxidising enzymes were :—

Sample I. The natural latex undiluted.

Sample II. One pint of latex treated with 2 tea-spoonfuls of ammonia (S.G. 0·880) in ½ oz. of water.

Sample I was a clear white milky fluid which had not coagulated at all. On suitable dilution the globules of caoutchouc on examination under the microscope were found to be intact and in rapid brownian movement in the serum. This sample of latex although uncoagulated had developed a marked acidity to litmus[2] which is perhaps surprising, in view of the generally accepted theory in regard to the process of coagulation.[3]

1. This latex was collected for me through the kindness of Mr. T. F. Burrowes, the Acting Colonial Secretary for Southern Nigeria, and I should like to take this opportunity of expressing my thanks to him and to those who have assisted him in securing for me an ample supply of latex for further investigations.

2. Fresh *Funtumia elastica* latex is practically neutral ! Experiments are already in progress which appear to show that the acidity developed in the natural latex is due to the oxydase therein.

3. This latex would indeed appear to be exceptionally stable in presence of acids ; see Spence, *Quart. Journ. Inst. Comm. Research*, Vol. II, No. 4, p. 45, 1907 ; Schidrowitz and Kaye, *India Rubber Journal*, Vol. XXXIV, No. 7, p. 377, 1907.

Sample II. This sample had not coagulated at all and was of a pale yellow colour in consequence of the presence in it still of a considerable excess of ammonia.

Both samples were tested directly with tincture of guaiacum with and without hydrogen peroxide. The first sample was found to give a very marked peroxydase reaction, but no reaction in absence of hydrogen peroxide.

To free the latex, however, from products which would tend to interfere with the peroxydase reactions, both samples of latex were dialysed for twenty-four hours against running water and then examined.

The sample of untreated latex from which the free acid had been removed in this way was still found to give a very marked peroxydase reaction but no oxydase.

The sample of ammoniacal latex on the other hand now gave an intense peroxydase and a much less marked though positive oxydase reaction. This was confirmed by a positive reaction against boiled controls using the tincture of guaiacum, the indophenol, the *p*-phenylenediamine and the pyrogallol tests as indicators.

The oxygenase in Sample I had, therefore, been more or less destroyed by the acidity developed. The question as to whether new oxygenase is formed on removal of the acid from the sphere of action still remains to be proved. Experiments so far have not shewn whether the oxygenase like the peroxydase will recover its activity, although it would be remarkable, were this not so, that samples of rubber prepared from the two dialysed latices in the ordinary way should darken in colour at the same rate and to the same degree.

Experiments were now set on foot in order to isolate the oxydase from the latex. For this purpose several methods of separating the caoutchouc in the latex without at the same bringing down the protein with it were tried, and although the method in the case of the acid latex was simple, it being only necessary to dilute the dialysed latex with several volumes of distilled water in a separating funnel, and to allow the fine flakes of caoutchouc to rise to the surface and settle before drawing off the mother-liquor, the process in the case of the

ammoniacal product was not so easy, there being a great tendency for the protein to disappear in the rubber clot. The following method for the ammoniacal latex was found to be satisfactory :—

The dialysed latex was diluted by its own volume with water and it was then treated with 50 per cent. alcohol until the agglutination of the caoutchouc particles was complete. The fine flakes of caoutchouc were allowed to settle, after which the mother-liquor was filtered off and treated with a large excess of absolute alcohol. On adding excess of alcohol a gummy precipitate formed which was immediately filtered off and dried in vacuo. This precipitate gave a marked oxydase reaction.

Properties of the Oxydases from the Latex

The product separated from the latex in this way was a dark brown vitreous-looking mass which dissolved only slowly in water, giving a somewhat opalescent solution. The aqueous solution was tested with the various reagents for oxydases both with and without hydrogen peroxide, and it was found to give a marked positive reaction *in absence of peroxide* and an intense coloration with the various reagents in presence of peroxide. It contained, therefore, the full oxydase complement (oxygenase-peroxydase) although the oxygenase appeared to be very feeble.

The enzyme was fairly stable towards heat and light in the dry state, but was readily destroyed in solution. It gave the xanthoproteic, Millon's, biuret, coagulation and precipitation tests for protein in a marked degree. It was also found to contain a considerable quantity of iron, and on fusion with alkali evolved ammonia and pyrrol, the latter being identified by the pinewood-HCl reaction already described. As in the case of the peroxydase from Para rubber the oxydase was also found to have associated with it a reducing substance which, from the violet red coloration (shewing an absorption band between D and E) given by it on warming in aqueous solution with an equal volume of HCl and a trace of phloroglucin, appears to be partly at least of the nature of a pentose. This being the case, it does not appear to me to be necessary or even correct to assume that a pyrrol ring is there *preformed* in the protein nucleus of the oxydase.[1] It seems far more probable that the pyrrol is formed

1. See Tschirch, 'Ueber die Gummi-Enzyme,' *Pharm. Zentralb.*, Nr. 31, 1905.

only in the process of fusion of the oxydase with the alkali, the ammonia liberated in this process reacting with the pentose or the partially oxidised pentose.[1] That such a reaction will actually result in the formation of a pyrrol derivative sufficient in amount to give a marked pinewood reaction, I have proved to myself to be the case by fusing together a mixture of pure arabinose, powdered potassium hydrate and ammonium sulphate. Although no coloration is given to the pinewood splint when the reaction is carried out in absence of ammonia, in the presence of this reagent I had no difficulty in getting, in repeated experiments, marked evidence of pyrrol formation.

The oxydase product isolated from the latex of *Funtumia elastica* did not appear to have proteolytic or amylolytic properties. It had a slight catalytic action.

In order to determine in how far the darkening in colour in rubber could be prevented practically by the removal of the nitrogenous products which form the 'insoluble constituent' of india-rubber, experiments were carried out with the Funtumia latex, the object being to separate the caoutchouc from the emulsion without at the same time coagulating the protein material in the latex. The following method was completely successful :—

The acid latex is diluted with many times its own volume of water in a separating funnel. The caoutchouc globules apparently coalesce in this way to form fine spongy flakes.[2] These flakes gradually separate and rise to the surface of the watery liquor, which is then drawn off. The flakes of caoutchouc are again shaken up with a fresh quantity of water, and when this process is repeated three times the caoutchouc is found to be practically nitrogen-free. The flakes are then worked up into a solid rubber clot by washing with alcohol or by pressure.

The rubber obtained in this way was snow-white and did not darken in the slightest on keeping. Samples prepared from the same latex by any of the usual methods of coagulation in which the protein is afterwards to be found in the rubber clot, darken rapidly in colour and become almost black in the course of a week. A sample of rubber prepared by the method described was found to be practically free from protein.[3]

1. Compare C. Paal, *Furfurane, thiophene and pyrrol syntheses*, Würzburg, 1890.
2. Coagulation does not take place.
3. The protein was found uncoagulated in the first washings. The value commercially of such a method of coagulation will be discussed elsewhere.

These observations prove conclusively that the darkening in colour of raw rubber is due to an oxydase which is associated with the protein or the so-called insoluble constituent of the rubber. In how far this oxydase is responsible for certain other changes occurring in raw rubber on keeping (decomposition, oxidation and the like), further experimental work, I hope, will shew.

In addition to its practical importance the presence of active oxydase enzymes in the latex of caoutchouc-producing plants appears to me to be of interest biologically when considered in the light of certain well-known facts regarding the chemistry of india-rubber. Up to the present no explanation of the function of the caoutchouc in the latex has been offered. The chemical inertness of india-rubber as we know it, and the fact that this product can be extracted in such quantities without any apparent injury to the tree, has led to the general belief that it is an excretory product of metabolism serving as a means of protection to the plant against injury[1] (*Wundverschluss*).

That the latex plays a useful part as a reserve store of water for the plant and as a means of protection against external injury, one need not doubt, but that these are the chief functions of the latex of caoutchouc-producing plants seems to me improbable. The economy and care with which the vital processes in nature are carried out and regulated, point on the very surface to a deeper and more important function for the caoutchouc in the plant, and it is scarcely to be accepted without further proof that a caoutchouc-producing tree continues to produce immense quantities of material in the nature of a hydrocarbon—the richest form of chemical energy that one can well imagine—solely as a means of protection against injury. From the very nature of the product, it seems to me we must far rather believe that it has a more important function in the plant, and there is considerable evidence on all hands to indicate this.

I would venture to suggest that the caoutchouc is probably a reserve food stuff for the plant, and to account for its formation and subsequent decomposition would bring the oxidizing enzymes under

1. See Czepek '*Biochemie der Pflanzen*,' Vol. II, pp. 698-701, 1905, and Tschirch, '*Die Harze und die Harzbehälter*,' 2nd edit., Vol. II, 1906.

consideration. Just as the glycogen in the liver is believed to be a reserve store of energy built up from the simpler sugars only to be broken down again into these and other products by the interaction of the glycolytic enzyme, so the caoutchouc may also be regarded as a reserve food stuff which is broken down by the oxydases in the plant as circumstances demand, into the simpler products from which it has been formed.

That the oxydases are capable of effecting such changes might appear at first sight hard to believe. But as evidence that such enzymes have already been shewn to bring about even more complicated changes, I should like to point out :—

The complete oxidation of sugar into CO_2 and H_2O by oxidizing enzymes has been shewn by the work of Sieber,[1] Palladin,[2] and others. The oxidation of complex fats into simpler fatty acids and CO_2 has been illustrated by the experiments of Tolomei[3] on the oxidation of olive fat by olease—an oxidation enzyme in olives giving a marked tincture of guaiacum reaction. Finally, the oxidation of the protein decomposition product tyrosin by means of an oxidizing enzyme (the tyrosinase of Bertrand[4]) into homogentisinic acid, carbon dioxide and ammonia with the uptake of oxygen is sufficient evidence, I think, to shew that in complicated oxidation processes in both the animal and the vegetable kingdom oxidizing enzymes play an important rôle.

Nor does the decomposition of caoutchouc by oxidizing enzymes in the plant appear to me to offer insurmountable difficulties in the way of the theory suggested. Certain observations already made on the action of a peroxydase enzyme and hydrogen peroxide on the neutral latex emulsion of *Funtumia elastica* seem to indicate that the caoutchouc in the latex is not indifferent towards atomic oxygen liberated in this way. Further, the important discovery of Harries,[5] that caoutchouc, even in the form in which it is known

1. Sieber, *Zeit. phys. Chem.*, Vol. XXXIX, p. 484, 1903.

2. Palladin, *Zeit. f. physiol. Chemie*, Vol XLVII, p. 407, 1906.

3. Tolomei, *Chem. Centr.*, Vol. I, p. 879, 1896.

4. Bourquelot and Bertrand, *Journ. pharm. chim* (6), Vol. 3, p. 177, 1896; Bourquelot, ***Bull. Soc. Mycol.***, France, p. 65, 1897; v. Furth and Schneider, *Hofmeister's Beiträge*, Vol. I, p. 229, 1901; v. Furth and Jerusalem, *Hofmeister's Beiträge*, Vol. X, p. 131, 1907, and numerous others.

5. Harries, *Ber. chem. Ges.*, Vol. XXXVII, pp. 2708-2711, 1904, and Vol. XXXVIII, pp. 1195-1203, 1905.

commercially, is exceedingly sensitive towards small quantities of ozone, and indeed can be broken down by this means into products very nearly related to the pentoses[1] from which the caoutchouc is most probably formed by the plant, lends support to the view that the caoutchouc in the latex must be regarded as a reserve stuff which can be drawn upon as required and broken down by the oxidases always associated with it into simple carbohydrate products of value as food stuffs.[2]

If we go further, however, and assume that the reaction brought about by the oxydase is a reversible one, then by means of it we can account not only for the breakdown, but also for the building up of the caoutchouc from the sugars (from the pentoses probably as Harries suggested) by the plant. In this light, the presence of a pentose group associated with the oxydase from the latex of *Funtumia elastica* is highly interesting.[3]

The theory suggested to account for the formation and the function of the caoutchouc in the latex is supported further by experimental facts regarding the anatomy and physiology of caoutchouc-producing plants. It would explain, for example, the occurrence of a definite latex system in the embryo of certain caoutchouc-producing plants, and the complete disappearance of the latex from the tree under certain conditions, but these aspects of the question will be considered in full when further experimental evidence on the chemical side has been obtained.

In the meantime, I should like to reserve to myself the right to follow up the work on which I have already commenced, of investigating the action of oxidizing enzymes on latex emulsions.

In conclusion, I beg to express my thanks to Professor Benjamin Moore for the interest which he has taken in the progress of the work.

1. Laevulinic acid and hydrogen peroxide.

2. The peculiar state of division of the caoutchouc in the latex, and the fact that it is there most probably in a less complex form than in the coagulated product must not be overlooked. See Weber, *Ber. chem. Ges.*, Vol. XXXVI, pp. 3108-3115, 1903; Harries, *Ber. chem. Ges.*, Vol. XXXVII, p. 3842-3848, 1904.

3. Whether the so-called impurity always associated with the various enzymes may not represent minute quantities of the substrate in loose chemical combination or adsorption with the enzyme, in the process of change, is a point worthy of further consideration. If this were so then the nature of the so-called impurity so firmly attached to the enzyme ought to give a valuable indication of the function of the latter.

ON THE APPLICATION OF BARFOED'S REAGENT TO SHOW THE HYDROLYSIS OF DISACCHARIDES BY ENZYMES

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The methods which are used to show the hydrolytic splitting of lactose and maltose, are not so simple as those which demonstrate the hydrolysis of saccharose and polysaccharides. The reason for this is that in the latter case there is a change from a non-reducing carbohydrate to a reducing sugar, whilst in the former the original material will already reduce cupric hydrate in alkaline solution. It is, therefore, necessary to make a quantitative estimation of the reducing power in two solutions, one of which is a control containing the boiled enzyme and the other is a similar mixture containing the unboiled enzyme.[1] The presence of lactase and maltase can be just as easily proved as the ordinary amyloclastic enzymes, by using copper acetate in acetic solution.[2] This reagent is reduced by monosaccharides, whilst under proper conditions, disaccharides leave it unchanged.

Hinkel and Sherman have recently investigated this reaction by comparing the behaviour of the reagent with glucose, maltose, lactose and saccharose.[3] They found that using five cubic centimetres of the reagent the presence of 0·0004 gramme glucose can be shown, either alone, or mixed with disaccharides, provided that the total weight of disaccharide does not exceed 0·02 gramme. The period

1. Polarimetric and other tests have also been used, see Aders Plimmer, *Journ. Physiol.*, Vol. XXXV, p. 20, 1907.

2. Barfoed, *Fresenius Zeit. f. anal. Chem.*, Vol. XII, p. 27, 1873.

3. *Journ. Amer. Chem. Soc.*, Vol. XXIX, p. 1744, 1907.

of heating should, however, not be too long or reduction may occur owing to hydrolysis of the disaccharide by the acetic acid in the reagent. They also recommend that each observer should standardise his reagent before using it.

Their observations have been confirmed by me, and it seemed desirable to apply this reagent as a qualitative test,[1] when testing for the presence of lactase and maltase.

To demonstrate the presence of lactase and maltase, the following method was employed. To five cubic centimetres of a one per cent. solution of the disaccharide, one cubic centimetre of an aqueous (toluol water) extract of the first part of the small intestine was added ; toluol being used as a preservative. After the period of digestion was ended, one cubic centimetre of this mixture was added to five cubic centimetres of the copper acetate reagent, and the resulting solution was placed in a boiling water bath. The tubes were examined at the end of three minutes, and if no reduction was visible they were replaced and re-examined at the end of the fourth and fifth minutes.

It was found that one cubic centimetre of a one per cent. solution of either lactose or maltose does not cause reduction until heated for nine or ten minutes. Thus, if the heating does not exceed five minutes any reduction with this strength of sugar must be due to the formation of monosaccharides. There is also a further check on the accuracy of the method, the control should show no reduction and as it is heated under identical conditions, the absence of reduction proves that the reduction caused by the other solution must be due to the hydrolytic action of an enzyme.

Experiment I.—Aqueous extract of duodenum and first portion of jejunum of cat.

Solution	At end of 1 day	At end of 3 days
5 c.c. 1 % lactose + 1 c.c. unboiled enzyme	No reduction	Slight reduction within 5 minutes
5 c.c. 1 % lactose + 1 c.c. boiled enzyme	No reduction	No reduction
5 c.c. 1 % maltose + 1 c.c. unboiled enzyme	Marked reduction within 3 minutes	—
5 c.c. 1 % maltose + 1 c.c. boiled enzyme	No reduction	—

1. This reagent might also be applied quantitatively, as glucose can be completely removed from a solution if the total amount does not exceed 0·002 g. per 5 c.c. of the reagent (Hinkel and Sherman, p. 1747) and the oxide separates in a form which could easily be filtered off and weighed.

Experiment II.—Aqueous extract of first portion of small intestine of a kitten four days old.

Solution		At end of 1 day
5 c.c. 1 % lactose + 1 c.c. unboiled enzyme	...	Marked reduction within 3 minutes
5 c.c. 1 % lactose + 1 c.c. boiled enzyme	...	No reduction
5 c.c. 1 % maltose + 1 c.c. unboiled enzyme	...	Marked reduction within 3 minutes
5 c.c. 1 % maltose + 1 c.c. boiled enzyme	...	No reduction

These two experiments show that both lactase and maltase were present in the intestine of a kitten whilst the adult cat possessed maltase, but the amount of lactase was much less than in a young animal.[1]

This result, agreeing as it does with previous workers who have studied the distribution of lactase and maltase, suggests that Barfoed's reagent may be used whenever it is wished to demonstrate the hydrolysis of disaccharides.

1. Cf. Plimmer, *loc. cit.*, p. 29.

A RAPID METHOD FOR SEPARATING HIPPURIC ACID FROM URINE

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When hippuric acid is precipitated by acid from a dilute solution of one of its salts it usually separates slowly, and when obtained from urine it also carries down with it a certain amount of pigment.[1]

The following method has been found a convenient way in which to prepare hippuric acid from (herbivorous) urine. It consists, in short, in adding ammonium sulphate to the urine before acidification. By this means the hippuric acid crystallises out rapidly, and usually contains only a comparatively small amount of adherent pigment.[2] This can be removed, and the crystals obtained free from pigment, by recrystallisation, after boiling the free acid (or its sodium salt) with animal charcoal.

The following experiments will illustrate the results which can be obtained by altering the conditions of experimentation. To samples of twenty-five cubic centimetres of cow's urine varying amounts of salt and acid were added, and the time at which crystallisation commenced was noted. In some cases the crystals were filtered off after a definite period and the filtrate allowed to stand for twenty-four hours to see if a further crop resulted.

1. For the usual methods of preparing hippuric acid see *Naubauer u. Vogel*, Harn Analyse, 10th ed. p. 225, 1899.

2. Previous treatment by heating with milk of lime and then filtering did not appear to lessen the amount of pigment in the crystals.

	Urine	Salt	Acid	Crystallisation commenced	Remarks
1	25 c.c.	—	1 c.c. 31 % HCl	—	Only one or two deeply pigmented crystals at the end of 24 hours
2	25 c.c.	—	1 c.c. 31 % H_2SO_4	—	
3	25 c.c.	6 g. NH_4Cl	1 c.c. 31 % H_2SO_4	—	No crystals recognisable at end of 24 hours
4	25 c.c.	6 g. $(NH_4)_2SO_4$	1 c.c. 31% H_2SO_4	15 mins.	Good yield in 35 mins., not complete in 1 hour
5	25 c.c.	6 g. $(NH_4)_2SO_4$	2 c.c. 31 % H_2SO_4	20 mins.	Tends to become pigmented, good yield
6	25 c.c.	6 g. $(NH_4)_2SO_4$	1 c.c. 98 % H_2SO_4	20 mins.	Slowly becomes pigmented, good yield
7	25 c.c.	8 g. $(NH_4)_2SO_4$	1 c.c. 31 % H_2SO_4	10 mins.	Good yield in 30 mins., not complete in 1 hour
8	25 c.c.	6 g. $(NH_4)_2SO_4$	1 c.c. 31 % HCl	15-20 mins.	Good yield in 30 mins., not complete in 1 hour
9	25 c.c.	6 g. $(NH_4)_2SO_4$	0·75 c.c. 31 % HCl	10 mins.	Not so good a yield as in No. 8
10	25 c.c.	6 g. $(NH_4)_2SO_4$	2 c.c. 31 %HCl	15 mins.	Crystallisation slow and incomplete, crystals pigmented
11	25 c.c.	8 g. $(NH_4)_2SO_4$	1 c.c. 31 % HCl	10 mins.	Good yield
12	25 c.c.	12 g. $(NH_4)_2SO_4$	1 c.c. 31 % HCl	5 mins.	Crystallisation complete in 10 mins.

From this table one sees that the addition of ammonium sulphate facilitates crystallisation, whilst ammonium chloride has no such effect. The greater the amount of ammonium sulphate the more rapid the crystallisation, and when saturation is approached the crystals are completely formed in ten minutes, so that on filtering them off no further crystallisation occurs, in the filtrate, even if it is allowed to stand for twenty-four hours. The amount and nature of the acid used has an influence on the rapidity of crystallisation. Using sulphuric acid it was found that increase of acid had practically no effect on the rate at which the crystals formed, but with hydrochloric acid it was found that increase of acid retarded the onset of crystallisation. Thus, the only disadvantage of adding excess of sulphuric acid is to cause an increase of pigmentation, whilst an excess of hydrochloric acid has the additional drawback of delaying crystallisation. It is, however, necessary to add sufficient excess of acid to completely separate hippuric acid from its salts (see experiments Nos. 8 and 9).

The delay of crystallisation caused by too large a quantity of hydrochloric acid was at first thought to be due to the hydrogen ion, but that idea is negatived by the experiments using larger quantities of sulphuric acid (Nos. 5 and 6). The true explanation

probably lies in some action of the chlorine ion, because a more marked delay is shown when ammonium chloride is substituted for ammonium sulphate (No. 3). This action is rather curious as it presents an analogy to the action of ammonium sulphate in precipitating proteins more readily than does ammonium chloride.

With large amounts of ammonium sulphate and not too large an amount of acid it naturally makes no difference whether hydrochloric or sulphuric acid is used. It is, however, safer to use sulphuric acid throughout the procedure so as to avoid any danger of delay by adding too large an amount of hydrochloric acid.

The best proportions for most purposes are 250 grammes ammonium sulphate (or an equal volume of saturated solution) and 15 c.c. concentrated (98 per cent.) sulphuric acid to each litre of urine. This mixture is allowed to stand for twenty-four hours and the crystals filtered off. When more rapid crystallisation is desired the amount of ammonium sulphate can be increased. The method has been found applicable to prepare hippuric acid from large volumes of urine and also, as a class experiment, to separate it and obtain its characteristic reactions using so small a quantity as 25 c.c. of urine.

A NEW COLORIMETRIC METHOD TO SHOW THE ACTIVITY OF EITHER 'PEPTIC' OR 'TRYPTIC' ENZYMES[1]

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Grützner, in 1874,[2] described a colorimetric method to estimate the amount of pepsin in a solution. He used fibrin which had been stained with carmine dissolved in ammonia. When the fibrin dissolved the carmine was set free and from the depth of colour the amount of fibrin which had been digested could be estimated. His method possesses one drawback in that it is applicable only to digestion in acid solution because when alkali is present the carmine is dissolved out of the fibrin without the latter being dissolved.

The stage of digestion which is shewn is the first step in hydrolysis, namely, that of the formation of acid-albumin. This stage, according to the present view of the action of proteoclastic enzymes in the body, is a very early one but the choice of it as an indicator of the rate of digestion is just as sound as many other methods which have been used. Almost any single stage might be taken when comparing the amount of the same enzyme in different solutions provided that it is regarded in the light of a means of indicating the rapidity of digestion and not as a final judgment on the normal mechanism of digestive processes.[3]

When one wishes to compare the relative activity of two enzymes, one of which acts better in an acid medium whilst the other only acts in an alkaline reaction, the stage of digestion chosen is of great importance. It is hardly fair to use a precipitant which precipitates

1. This method was shewn at a meeting of the Lancashire and Cheshire Branch of the British Medical Association, *British Medical Journal*, Vol. I, 1908, *Supplement*, p. 138.

2. *Arch. f. d. ges. Physiol.*, Vol. VIII, p. 452, 1874.

3. Cf. Bayliss, *Journ. Physiol.*, Vol. XXXVI, p. 225, 1907.

the end products of the one whilst the other, carrying the hydrolysis a step further, furnishes substances which remain in solution. A Kjeldahl nitrogen estimation of the filtrate after such a precipitation might shew entirely different results to a similar estimation made after the use of a different precipitant. There is, however, a certain amount of truth in saying that the further the hydrolytic splitting is carried the 'stronger' the digestive activity; but on the other hand there is something to be said for choosing, as a mark of the activity of a digestive enzyme, the rapidity with which an insoluble protein is dissolved.

The following method was devised to obviate the delay necessarily caused in carrying out total nitrogen estimations when a large number of comparative estimations are being made. It is brought forward in the belief that for most purposes it gives almost as much information as more elaborate procedures. The process is as simple as the 'Grützner' method, and possesses the advantage of acting just as well for digestions in alkaline solutions as for those in the presence of acid.

In seeking for some dye that would stain fibrin so that it would part with its colour neither to acid nor to alkali the one finally chosen was congo red. Bayliss[1] found that this dye is well absorbed and retained by filter paper, and that heating to 100° C. fixed the dye so that it cannot be removed by washing.

Fibrin stained by congo red was found to slowly part with its pigment when kept for some hours at 40° C. in the presence of one per cent. sodium carbonate. It is difficult to say whether this was due to slow solution of the fibrin or to the alkali removing the congo red from the fibrin, leaving the latter unchanged. This difficulty was surmounted by dropping the fibrin after staining into boiling water for a few minutes and then removing the excess of dye by washing in running water. Fibrin treated by this method did not cause any coloration of either 0·18 per cent. hydrochloric acid[2] or of

1. This Journal, Vol. I, p. 175, 1906.

2. The fibrin becomes changed from red to blue by the action of the acid.

1 per cent. sodium carbonate, when kept for one hour at 40° C. and then left at room temperature for three weeks.

As the heating to 100° C. renders fibrin less easily digested, the following experiment was performed to see if a lower temperature would suffice to prevent a red colour appearing in 1 per cent. sodium carbonate solution.

Experiment I.—20 gms. of moist fibrin, which had previously been minced and washed, was kept for 24 hours in 40 c.c. of 0·5 % solution of congo red. At the end of this time it was divided into six equal portions, and these were heated for five minutes to different temperatures. Each lot was then well washed and divided into two portions, one of which was placed in 0·18% HCl and the other in 1% Na_2CO_3 and they were all placed in the incubator at 40° C., toluol being used as a preservative.

Procedure	Kept at 40° C. in 0·18 % HCl At end of 2 hours	Kept at 40° C. in 0·18 % HCl At end of 24 hours	Kept at 40° C. in 1 % Na_2CO_3 At end of 2 hours	Kept at 40° C. in 1 % Na_2CO_3 At end of 24 hours
Heated for 5 mins. to 50° C.	No coloration	Bluish purple	Faint pink colour	Pink colour
Heated for 5 mins. to 60° C.	No coloration	Faint bluish purple	No coloration	Slight pink colour
Heated for 5 mins. to 70° C.	No coloration	Very faint bluish purple	No coloration	Slight pink colour
Heated for 5 mins. to 80° C.	No coloration	No coloration	No coloration	Slight pink colour
Heated for 5 mins. to 90° C.	No coloration	No coloration	No coloration	Slight pink colour
Heated for 5 mins. to 100° C.	No coloration	No coloration	No coloration	Slight pink colour

From this table it is seen that it is sufficient to keep the stained fibrin for five minutes at a temperature much lower than 100° C. in order to prevent the colour being removed by sodium carbonate. This appears to take place at 60° C., and is probably due to the destruction of an autolytic enzyme. As the final colour in all, except the first, was about the same tint, the slight colour observed is most probably due to a slight formation of alkali albumin by the long continued action of the carbonate at 40° C. In preparing the congo red fibrin it is a great advantage to use as low a temperature as possible because when the temperature has been raised to 100° C. for some time the fibrin is rendered much less easy to digest.

As the congo red turns blue with acid it is necessary to render the solution alkaline when it is desired to compare the depth of colour of an acid digest with that resulting from digestion in an alkaline solution. It was found that the easiest way to do this was to add solid anhydrous sodium carbonate until the precipitate, which forms

at the neutral point, redissolves. The depth of red colour of the product of peptic digestion can then be directly compared to that of the tryptic digest. Even when comparing peptic digestions with each other it is more convenient to render them alkaline before comparing them.

The method was next tested to compare whether the amount of colour given to the solution on digestion showed any relation to the amount of soluble nitrogen left after precipitation by trichloracetic acid.

Experiment II.—Two enzyme solutions, one peptic and the other tryptic, were prepared, and the rate of digestion was compared as follows :—Twenty cubic centimetres of the peptic enzyme was digested, for one hour at 40° C., with 1 gr. minced fibrin. This was then precipitated by ten cubic centimetres of a ten per cent. solution of trichloracetic acid and filtered hot ; twenty-five cubic centimetres of the filtrate was then Kjeldahled. The number of cubic centimetres of decinormal acid given by a control, in which the enzyme had been destroyed by boiling before digestion, was subtracted from the figure obtained for the peptic digest, giving 7·4 c.c. The figure given for the tryptic enzyme under similar conditions was 8·0 c.c. The ratio of tryptic to peptic digestion was thus found to be 1·1.

Ten cubic centimetre portions of the same enzyme solutions were compared by the method described in this paper, namely by adding 0·5 gr. congo red fibrin and diluting the digest, obtained by allowing digestion to proceed for one hour at 40° C., until the solutions were of equal tint. The ratio obtained by this method was 1·0.

A second experiment, using different enzyme solutions, gave the following ratio of tryptic to peptic action. From total nitrogen determinations, 1·3. From congo red dilution, 1·5.

It is not claimed that the method of diluting the solutions until the colours appear to be the same is very accurate, but the results show a certain parallel between the two methods employed.

The relation, of the amount of enzyme added, to the rate of solution was also investigated.

Experiment III.—

Ratios of peptic enzyme in 10 c.c. of 0·18 % HCl with 0·5 gr. congo red fibrin	Ratios of enzyme action as shown by the amount of dilution to reach an equal tint	Ratios of tryptic enzyme in 10 c.c. of 1 % Na_2CO_3 with 0·5 gr. congo red fibrin	Ratios of enzyme action as shown by the amount of dilution to reach an equal tint
4	2	4	4
9	2.9	9	10
16	3.6	16	18

This experiment shows that the enzyme action, as shewn by solution of congo red fibrin, for pepsin follows the Schütz[1] law as it is nearly the square root of the amount of enzyme present, whilst for trypsin it is roughly proportional to the amount of enzyme present.

It is thus shown that fibrin stained by congo red can be used to shew the amount of either peptic or tryptic enzyme present in a solution. The best method to prepare the fibrin is as follows. The fibrin should be minced and washed until free from blood. To each hundred cubic centimetres of one half per cent. solution of congo red in water, add fifty grammes of moist fibrin. Leave for twenty-four hours and then pour the pasty mass into a large volume of water heated to the required temperature and kept at that temperature for about five minutes. The temperature used can be varied according to the object required, but for most purposes 80° C. is the best, as it fixes the dye without interfering to any great extent with the ease of digestion of the resulting congo red fibrin. The fibrin can then be placed in a piece of cloth and washed in running water by tying the cloth on the nozzle of a tap and letting the water run slowly. After washing, the excess of water is squeezed out and the fibrin kept in equal parts of glycerine and water. When the fibrin is to be kept for a long time, a little toluol (or other preservative) can be added to prevent the growth of moulds.

Specimens of fibrin prepared in this manner have been found not to deteriorate on keeping, and one sample was found after an interval of five months to be just as good as when freshly prepared.

1. *Zeit. f. physiol. Chem.*, Vol. IX, p. 577, 1885.

ON THE FORMATION OF LACTIC ACID AND CARBONIC ACID DURING MUSCULAR CONTRACTION AND RIGOR MORTIS

By P. W. LATHAM, M.D., *Downing Professor of Medicine in the University of Cambridge* (1874-1894).

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When living muscular tissue is at rest its reaction to test paper is faintly alkaline or neutral. When it contracts, and during the period of rigor mortis, carbonic acid is set free, and the reaction of the muscle to test paper becomes distinctly acid. This acidity is supposed to result from the liberation of lactic acid; but in what way this acid and carbonic acid are produced, or from what proteid constituent they arise has not hitherto been determined.

Carbonic acid cannot be directly combined with lactic acid $CH_3 \cdot CH(OH) \cdot COOH$; the synthesis, however, may be effected by adopting the following method.

Pour ten grammes of pyruvic acid $CH_3 \cdot CO \cdot COOH$ very slowly and gently over some finely powdered potassium cyanide, then add slowly 12 c.c. of concentrated hydrochloric acid; pour the whole into a flask containing a hot solution of twenty grammes of barium hydrate in 250 c.c. of water; attach a reversible condenser and boil for two hours, neglecting any immediate precipitate which may be formed. After cooling collect the white crystalline salt on a filter. This salt, which is barium methyl tartronate, when dried at 100 °C., weighs about 16 grammes and contains only a very small quantity of barium carbonate.[1]

The following is the reaction :—

$$\underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH} + KCN = CH_3 \cdot C(OH \cdot CN)COOK$$

$$CH_3 \cdot C(OH \cdot CN) \cdot COOK + HCl = KCl + CH_3 \cdot C(OH \cdot CN) \cdot COOH$$

$$CH_3 \cdot C(OH \cdot CN) \cdot COOH + BaH_2O_2 = \underset{\text{barium methyl tartronate}}{CH_3 \cdot C(OH) \cdot (COO)_2Ba} + NH_3$$

1. C. Böttinger, *Berichte d. deutsch. chem. Gesellsch.*, Bd. XVII, s. 144.

On carefully adding to the barium methyl-tartronate the molecular equivalent of dilute sulphuric acid, methyl-tartronic acid, $CH_3 \cdot C(OH) \cdot (COOH)_2$ or iso-malic acid is liberated, and on evaporating the solution, rhombohedral crystals are obtained similar in appearance to those of Iceland spar.[1]

On heating methyl tartronic acid with hydrochloric acid it is resolved into carbonic acid and fermentation or α lactic acid ;

$$\underset{\text{methyl-tartronic or iso-malic acid}}{CH_3 \cdot C(OH) \cdot (COOH)_2} = CO_2 + \underset{\alpha \text{ lactic acid}}{CH_3 \cdot CH(OH) \cdot COOH}$$

Methyl-tartronic acid, however, is not one of the constituents of proteid matter, but I suggest that its antecedents or generators are ; and I shall endeavour to show that this hypothesis is true by considering :—

First, what products are formed in plants in the earliest stages in the synthesis of organic matter, and

Secondly, what products are furnished by the hydrolytic decomposition of proteid matter.

The Genesis of Organic Matter in Plants

The view which is now most generally accepted is that in plants CO_2 entering by the leaves combines with H_2O absorbed by the roots and from these glucose is said to be formed, a volume of oxygen equal to that of the CO_2 absorbed being exhaled by the plant ; the initial product being formaldehyde. This hypothesis was first advanced by A. von Baeyer.[2] MM. Loew and Bokorny and Pringsheim have shown that in living plasma there is a substance which has the property of reducing silver salts, and for this reason is regarded as an aldehyde ; the aldehyde resulting from the combination in the plant of CO_2 and H_2O. Pringsheim gives the following as the reaction :—

$$CO_2 + H_2O = \underset{\substack{\text{methylic} \\ \text{aldehyde}}}{H \cdot CHO} + O_2$$

1. Böttinger, *loc. cit.*, p. 148.
2. Berichte, Bd. III, s. 163.

It has long been a subject of interest to discover how, by purely chemical methods, this reduction could be carried out, and various investigators have from time to time endeavoured, but unsuccessfully, to solve the problem. Maly[1] in 1865, Lieben,[2] Ballo,[3] and others attempted it and were able to reduce the carbonates to formates, but no further. Recently, however, Dr. H. J. H. Fenton[4] has succeeded by means of magnesium in reducing carbon dioxide to formaldehyde at a low temperature in aqueous solutions, demonstrating the truth of the above assumption. Acted upon by the contents of the living cell[5] condensation of the aldehyde takes place, as is the case when it is acted upon by alkalies, and glucose is formed :

$$\underset{\text{aldehyde}}{6H{\cdot}CHO} = \underset{\text{glucose}}{C_6H_{12}O_6}$$

which by dehydration is converted according to circumstances into starch, cellulose or glucoside :

$$C_6H_{12}O_6 = \underset{\text{starch}}{C_6H_{10}O_5} + H_2O$$

If, on the other hand, formaldehyde is oxidised in the leaves of the plant it is converted into formic acid :

$$\underset{\text{formaldehyde}}{H{\cdot}CHO + O} = \underset{\text{formic acid}}{H{\cdot}COOH}$$

which now combines with ammonia absorbed by the roots to form ammonium formate, which then, by dehydration, is converted into hydrocyanic acid :

$$\underset{\text{formic acid}}{H{\cdot}COOH} + NH_3 = \underset{\text{ammonium formate}}{H{\cdot}COO{\cdot}NH_4}$$

$$= \underset{\text{prussic acid}}{HCN} + 2H_2O$$

1. *Liebig Annalen*, Bd. CXXXV, s. 119.
2. *Monatschr.*, Bd. XVI, s. 211, and Bd. XVIII, s. 582.
3. *Berichte*, Bd. XVII, s. 6.
4. *Transact. of the Chemical Society*, Vol. XCI, p. 687, 1907.
5. Adolph Baeyer, *Berichte*, Bd. III, s. 68.

The prussic acid may now react in various ways, as follows :—

A.—By oxidation it is converted into cyanic acid

$$HCN + O = H{\cdot}OCN$$

B.—By reduction it is converted into methylamine

$$H{\cdot}CN + H_4 = CH_3{\cdot}NH_2$$

C.—It may combine directly with formic aldehyde and produce methyl cyan-alcohol or glycollic nitrile

$$H{\cdot}CHO + HCN = CH_2OH{\cdot}CN$$

which combined with $2H_2O$ leads to glycollic acid and ammonia

$$CH_2{\cdot}OH{\cdot}CN + 2H_2O = \underset{\text{glycollic acid}}{CH_2{\cdot}OH{\cdot}COOH} + NH_3$$

By means of a reducing agent this acid may be converted into acetic acid[1]

$$CH_2{\cdot}OH{\cdot}COOH + H_2 = \underset{\text{acetic acid}}{CH_3{\cdot}COOH} + H_2O$$

the latter in combination with formic acid giving rise to acetic aldehyde

$$CH_3{\cdot}COOH + H{\cdot}COOH = \underset{\text{acetic aldehyde}}{CH_3{\cdot}CHO} + CO_2 + H_2O$$

or by dehydration to acetic anhydride

$$2CH_3{\cdot}COOH = (CH_3{\cdot}CO)_2\,O + H_2O$$

Acetic aldehyde and acetic anhydride may also be obtained by another method from glycollic nitrile

$$CH_2{\cdot}OH{\cdot}CN + H_6 \rightarrow CH_3{\cdot}CH_2{\cdot}NH_2 \rightarrow CH_3{\cdot}CH_2{\cdot}OH$$
$$\rightarrow CH_3{\cdot}CHO \rightarrow CH_3{\cdot}COOH \rightarrow (CH_3{\cdot}CO)_2O$$

By a similar series of changes we can then proceed to the formation of higher aldehydes, propionic, butyric, valerianic, etc., their acids and anhydrides.

1. Claus, *Annalen d. Chemie*, Bd. CXLV, s. 256.

By combining glycollic nitrile with NH_3 and subsequent hydration we obtain glycocoll[1]

$$CH_2(OH)\cdot CN + NH_3 = CH_2(NH_2)\cdot CN + H_2O$$
$$CH_2(NH_2)\cdot CN + 2H_2O = \underset{\text{glycocoll}}{CH_2(NH_2)\cdot COOH} + NH_3$$

In the same way by combining it with methylamine we obtain sarcosine

$$CH_2(OH)\cdot CN + NH_2(CH_3) = CH_2(NH\cdot CH_3)\cdot CN + H_2O$$
$$CH_2(NH\cdot CH_3)\cdot CN + 2H_2O = \underset{\text{sarcosine}}{CH_2(NH\cdot CH_3)\cdot COOH} + NH_3$$

which combined with cyanamide forms kreatin.

D.—The most interesting and important action, however, is the formation of amino-malonic nitrile from the condensation of three molecules of prussic acid

$$3HCN = \underset{\text{amino-malonic nitrile}}{CH(NH_2)\cdot(CN)_2}$$

Little attention has been paid to this substance, but I have reason to suppose that it plays an important part in the synthesis of the various proteids and of their derivatives. It is prepared in the following manner :—

If a fragment of potassium cyanide be added to anhydrous hydrocyanic acid, condensation of three molecules gradually takes place,[2] forming amino-malonic nitrile, a crystalline body

$$3HCN = \underset{\text{amino-malonic nitrile}}{CH(NH_2)\,(CN)_2}$$

a black insoluble substance being produced at the same time. The nitrile may be extracted with ether ; this, on evaporation, yields the crystals, which may then be dissolved in water, decolourised with animal charcoal, and recrystallised. The yield by this method, however, is small. A much larger result, not less than 50 per cent. of the theoretical amount, is obtained by the following process.[3]

1. Eschweiler, *Annalen d. Chemie*, Bd. CCLXXVIII, s. 655.
2. Lescoeur, Regault, *Bulletin de la Société Chimique de Paris*, t. XLIV, p. 473.
3. Eug. Bamberger, Leo. Rudolph, *Berichte*, Bd. XXV, s. 1083.

Add 2·6 grammes of pure dimethyl-aniline-oxide to 0·6 gramme of anhydrous prussic acid. Let the pale yellow mixture remain in the dark for twenty-four hours at the ordinary temperature. Extract with boiling water and separate the nitrile from the aqueous extract by shaking it several times with ether. It must then be recrystallised from boiling water and decolourised with animal charcoal.

Warmed with barium hydrate, amino-malonic nitrile is resolved into glycocoll, ammonia, and carbonic acid

$$\underset{\text{amino-malonic nitrile}}{CH(NH_2)\cdot(CN)_2} + 4H_2O = \underset{\text{glycocoll}}{CH_2(NH_2)\cdot COOH} + CO_2 + 2NH_3$$

the carbonic acid and ammonia being liberated in the same proportions as result from the decomposition of urea when heated with barium hydrate.

The intermediate stages may be represented as follows :—

$$CH(NH_2)\cdot(CN)_2 + 2H_2O = CH(NH_2)\cdot\left\langle\begin{matrix} CO\ NH_2 \\ CO\ NH_2 \end{matrix}\right.$$

which with a further molecule of H_2O

$$= CH_2(NH_2)\cdot COOH + CO\left\langle\begin{matrix} NH_2 \\ NH_2 \end{matrix}\right.$$

or, if we suppose that the NH_3 and CO_2 are discharged separately we may assume that they combine to form ammonium carbamate since the two gases if passed in a perfectly dry state into cold absolute alcohol are known to combine, the product separating as a copious crystalline precipitate. This, if filtered off and then heated with absolute alcohol in a sealed tube to 100°, on cooling appears as large crystalline laminae of ammonium carbamate.[1] Under certain conditions this is converted into urea and ammonium carbonate

$$CO_2 + 2NH_3 = \underset{\text{ammonium carbamate}}{NH_2\cdot COONH_4}$$

$$2NH_2\cdot COONH_4 = \underset{\text{urea}}{CO\left\langle\begin{matrix} NH_2 \\ NH_2 \end{matrix}\right.} + \underset{\text{ammonium carbonate}}{(NH_4)_2CO_3}$$

1. Kolbe and Basaroff, *Chem. Soc. Journal*, [2] 6, 194.

If glycocoll be heated with acetic anhydride it is converted into acetyl-glycocoll.[1]

$$\underset{\text{Glycocoll}}{2CH_2\genfrac{}{}{0pt}{}{\diagup NH_2}{\diagdown COOH}} + \underset{\text{Acetic anhydride}}{(CH_3{\cdot}CO)_2O} = \underset{\text{Acetyl-glycocoll}}{2CH_2\genfrac{}{}{0pt}{}{\diagup NH{\cdot}CO{\cdot}CH_3}{\diagdown COOH}} + H_2O$$

If it be heated in a sealed tube to 160° C. with benzoic acid it is converted into hippuric acid[2]

$$\underset{\text{glycocoll}}{CH_2\genfrac{}{}{0pt}{}{\diagup NH_2}{\diagdown COOH}} + \underset{\text{benzoic acid}}{C_6H_5{\cdot}COOH} = \underset{\text{hippuric acid}}{CH_2\genfrac{}{}{0pt}{}{\diagup NH{\cdot}CO{\cdot}C_6H_5}{\diagdown COOH}} + H_2O$$

which is a normal constituent of the urine of herbivorous animals, and which appears also in human urine after the administration of benzoic acid.[3]

In the *Lancet* for December 16, 1905, and December 8, 1906, I described a new method for the synthesis of tyrosine from amino-malonic nitrile (that is from prussic acid) and oxy-benzaldehyde, the following being the reactions :—

$$\underset{\text{amino-malonic nitrile}}{NH_2{\cdot}CH(CN)_2} + 4H_2O = \underset{\text{glycocoll}}{CH_2(NH_2){\cdot}COOH} + CO_2 + 2NH_3$$

$$\underset{\text{glycocoll}}{CH_2(NH_2)COOH} + \underset{\text{benzoic acid}}{C_6H_5{\cdot}COOH} = \underset{\text{hippuric acid}}{CH_2(NH{\cdot}CO{\cdot}C_6H_5){\cdot}COOH} + H_2O$$

$$\underset{\text{p. oxy-benzaldehyde}}{OH{\cdot}C_6H_4{\cdot}CHO} + \underset{\text{hippuric acid}}{CH_2(NH{\cdot}CO{\cdot}C_6H_5){\cdot}COOH} =$$

$$\underset{\text{oxy-benzoyl amino-cinnamic acid}}{OH{\cdot}C_6H_4{\cdot}CH : C(NH{\cdot}CO{\cdot}C_6H_5){\cdot}COOH} + H_2O$$

This heated in a sealed tube with 2KCN to 175°-182° is converted into

$$\underset{\text{tyrosine}}{p.\ OH{\cdot}C_6H_4{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH},\quad \underset{\text{ammonium benzoate}}{C_6H_5{\cdot}COO{\cdot}NH_4},\quad \text{and } \underset{\text{potassium oxalate}}{(COO)_2K_2}$$

the 2KCN acting as the reducing agent ; when heated with $4H_2O$ being converted into

$$H_2 + 2NH_3 + (COO)_2K_2$$

1. Curtius, *Berichte*, Bd. XVI, s. 757, and Bd. XVII, s. 1663.
2. Dessaignes, *Jahresbericht der Chemie*, s. 367, 1857.
3. Bouis, Ure : *Berz. Jahresbericht*, Bd. XXII, s. 567.

If, instead of using hippuric acid in the above synthesis, acetyl glycocoll, $CH_2(NH{\cdot}COCH_3)COOH$, is used, the same series of reactions will ensue, ammonium acetate instead of the benzoate being among the final products.

This experiment suggests that HCN; $CH(NH_2){\cdot}(CN)_2$; $(CH_3{\cdot}CO)_2O$; together with $OH{\cdot}C_6H_4{\cdot}CHO$ (or very probably $NH_2{\cdot}C_6H_4{\cdot}CHO$) are among the earliest products formed in the synthesis of proteid.

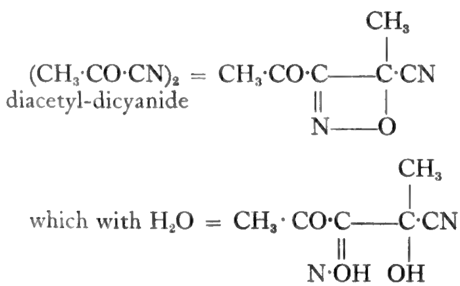
We have now to consider in what way we can synthesise methyl tartronic nitrile and its amino compound, namely, iso-aspartic or amino-iso-succinic acid from these products.

By mixing together 16 c.c. of acetic anhydride and 74 c.c. of dry ether, placing the solution in an ice bath and after slowly adding 10 grammes of KCN, passing through the mixture a stream of HCl gas and leaving it at rest in a bath at 0° for twenty-four hours, we obtain diacetyl-dicyanide $(CH_3{\cdot}CO{\cdot}CN)_2$,[1] the polymeride of pyruvic nitrile $CH_3{\cdot}CO{\cdot}CN$, or acetyl cyanide

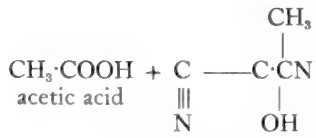
$$\underset{\text{acetic anhydride}}{(CH_3{\cdot}CO)_2O} + 2KCN + 2HCl = (CH_3{\cdot}CO)_2{\cdot}O + 2HCN + 2KCl$$

$$= \underset{\text{diacetyl-dicyanide}}{(CH{\cdot}CO{\cdot}CN)_2} + H_2O + 2KCl$$

Diacetyl-dicyanide is readily soluble in alcohol, ether and benzol. If left at rest with fuming HCl for twenty-four hours and then boiled with dilute HCl it is transformed into acetic acid and iso-malic or methyl-tartronic acid. According to L. Bouveault[2] the following are the reactions :



1. Brunner, *Monatsheft. f. Chemie*, Bd. XIII, s. 835.
2. *Bulletin de la Soc. chimique* [3] t. IX, p. 557.



methyl-tartronic nitrile

the latter being immediately saponified into methyl-tartronic acid and $2NH_3$.

Judging from analogy, however, methyl-tartronic nitrile should also result from the combination of diacetyl-dicyanide with 2HCN ;

$$\underset{\text{diacetyl-dicyanide}}{(CH_3{\cdot}CO{\cdot}CN)_2} + 2HCN = \underset{\text{methyl-tartronic nitrile}}{2CH_3{\cdot}C(OH){\cdot}CN)_2}$$

which by hydration is resolved into lactic acid, CO_2 and $2NH_3$

$$\begin{aligned} \underset{\text{methyl-tartronic nitrile}}{CH_3{\cdot}C(OH){\cdot}(CN)_2 + 4H_2O} &= \underset{\text{methyl-tartronic or iso-malic acid}}{CH_3{\cdot}C(OH){\cdot}(COOH)_2} + 2NH_3 \\ &= \underset{\alpha\ \text{lactic acid}}{CH_3{\cdot}CH(OH)COOH} + CO + 2NH \end{aligned}$$

or, the CO_2 and NH_3 in the latter case being liberated in the same proportions that result from the decomposition of urea—we may have

$$CH_3{\cdot}C(OH){\cdot}(CN)_2 + 3H_2O = \underset{\alpha\ \text{lactic acid}}{CH_3.CH(OH){\cdot}COOH} + \underset{\text{urea}}{CO\left\langle\begin{matrix}NH_2\\NH_2\end{matrix}\right.}$$

If, when the methyl-tartronic nitrile is decomposed, the intermediate products, methyl-tartronic acid and ammonia being formed, other substances are present such as the ketonic acids or the cyan-alcohols, $R{\cdot}CH(OH){\cdot}CN$, the liberated NH_3 will combine with them and on the decomposition of the methyl-tartronic acid, α lactic acid, and CO_2 will be alone set free. This I suggest is the action which takes place during muscular contraction, an explosive outburst of the two products being the result : the NH_3 combining with other constituents of the muscular tissue.

The same products may be formed slowly when the muscle is at rest ; or if, when at rest, NH_3 is set free from some other constituent of the muscular tissue the NH_3 will combine with the methyl-tartronic

nitrile forming a compound homologous with amino-malonic nitrile, namely, amino-iso-succinic nitrile, which by hydration can be converted into *a* alanine or amino-propionic acid and urea—

$$\underset{\text{methyl-tartronic nitrile}}{CH_3{\cdot}C(OH){\cdot}(CN)_2} + NH_3 = CH_3{\cdot}C(NH_2){\cdot}(CN)_2 + H_2O$$

$$\text{and } \underset{\text{amino-iso-succinic nitrile}}{CH_3{\cdot}C(NH_2){\cdot}(CN)_2} + 3H_2O = \underset{a \text{ alanine}}{CH_3{\cdot}CH_3{\cdot}CH(NH_2)COOH} + \underset{\text{urea}}{CO\begin{cases}NH_2\\NH_2\end{cases}}$$

or the intermediate products amino-iso-succinic acid and NH_3 may be formed.

Before proceeding to prove that methyl-tartronic nitrile (or its components) are constituents of fibrin there are some extremely interesting points regarding amino-malonic nitrile, and its derivative tartronic nitrile, which are worthy of reference and consideration.

By des-aminating amino-malonic nitrile in the usual way, that is by means of HNO_2, it will be converted into tartronic nitrile

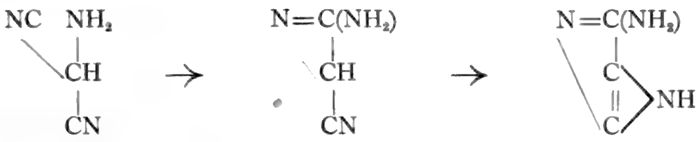
$$\begin{array}{l}CH\begin{cases}CN\\NH_2\end{cases}\\ \mid\\ CN\end{array} + H_2O = \begin{array}{l}CH\begin{cases}CN\\OH\end{cases}\\ \mid\\ CN\end{array} + NH_3$$

with which the nitriles of malic acid $\begin{array}{l}CH\begin{cases}CN\\OH\end{cases}\\ \mid\\ CH_2{\cdot}CN\end{array}$ and of oxyglutaric acid $\begin{array}{l}CH\begin{cases}CN\\OH\end{cases}\\ \mid\\ CH_2{\cdot}CH_2{\cdot}CN\end{array}$ are homologous.

In a later communication I shall endeavour to prove that these three nitriles are constituents of living proteid, being the precursors of amino-malonic, aspartic and glutamic acids, and that in dead proteid they are transformed into the anhydrides

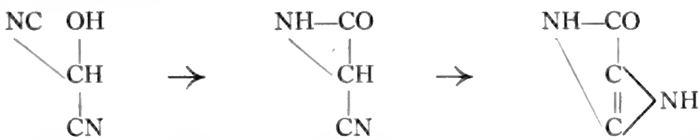
$$CN{\cdot}CH\begin{cases}NH\\ \mid\\ CO\end{cases},\quad CN{\cdot}CH_2{\cdot}CH\begin{cases}NH\\ \mid\\ CO\end{cases}\quad \text{and } CN{\cdot}(CH_2)_2{\cdot}CH\begin{cases}NH\\ \mid\\ CO\end{cases}$$

I further suggest that amino-malonic nitrile $CN{\cdot}CH\left\langle\begin{matrix}CN\\NH_2\end{matrix}\right.$ is a constituent of living proteid, which in dead proteid is transformed as follows—



amino-malonic nitrile

and that tartronic nitrile may be similarly transformed



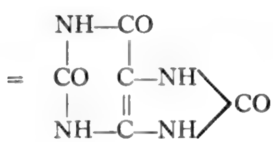
tartronic nitrile

and that if each of these is combined with 2HCN it is converted respectively into adenine and hypoxanthine

adenine

hypoxanthine

the latter when oxidised in the tissues being converted into uric acid[1]



1. Burian, R., *Zeitsch. f. physiol. Chemie*, Bd. XLI.

Another point of interest with regard to $CN\cdot CH\left<\begin{array}{l}NH\\ \;|\\ CO\end{array}\right.$ is that by reduction and hydration it can be converted into diamino propionic acid

$$CN\cdot CH\left<\begin{array}{l}NH\\ \;|\\ CO\end{array}\right. + H_4 = CH_2\cdot(NH_2)\cdot CH\left<\begin{array}{l}NH\\ \;|\\ CO\end{array}\right.$$

$$\text{and}\quad CH_2(NH_2)CH\left<\begin{array}{l}NH\\ \;|\\ CO\end{array}\right. + H_2O = \underset{\text{diamino propionic acid}}{CH_2\cdot NH_2\cdot CH(NH_2)COOH}$$

a body 'related chemically to a whole series of physiologically very important substances.' In the animal system this is transformed into glyceric acid[1]

$$CH_2\cdot(OH)\cdot CH\left<\begin{array}{l}OH\\ COOH\end{array}\right.$$

and this by reduction is converted into glyceric aldehyde

$$CH_2\cdot(OH)\cdot CH(OH)\cdot CHO$$

I have thus endeavoured to shew how, in the earlier stages of the genesis of organic matter the formation or synthesis of amino-malonic nitrile, and methyl-tartronic nitrile, may take place. What proof have I that these compounds are constituents of the proteid molecule ?

The proof rests upon the evidence furnished by the hydrolysis of albumin.

In 1875, P. Schutzenberger[2] communicated to the Academy of Sciences of Paris his first paper on the products obtained when albumin and its congeners are decomposed by simple hydration. This paper is a remarkable one, for had Schutzenberger been able rightly to interpret the results of his experiments the synthesis of albumin would, I believe, have been numbered among his achievements.

1. P. Mayer, *Zeitsch. f. physiol. Chem.*, Bd. XLII, s. 59.
2. *Comptes Rendus*, t. LXXX, p. 232.

Unfortunately he adopted an erroneous molecular formula for albumin (Lieberkühns), an error which he corrected a few years later. He also committed himself to the statement 'that the albuminous molecule contains an urea group, and represents a complex ureide,' which is not entirely borne out by the facts and has probably discouraged many from carefully considering the results which he obtained. These experiments, as recorded in his first paper, consisted in heating 100 grammes of coagulated albumin with 200 grammes of crystallised hydrate of baryta and one litre of water

(i) to a temperature of 100° C. under ordinary atmospheric pressure for 120 hours,

(ii) digesting them in an autoclave at a temperature of 140-150°C.

He determined in each experiment the amount of NH_3 liberated, and, from the amount of $BaCO_3$ formed, calculated the quantity of CO_2.

In both cases the amounts of CO_2 and NH_3 liberated were in the same proportion as those obtained from the decomposition of urea.

If Schutzenberger had been dealing only with such substances as amino-malonic nitrile, tartronic nitrile, methylamino, and methyl-tartronic nitriles his inferences would have been correct, but in his later experiments, in which the decomposition of albumin was more profound, this relationship between CO_2 and $2NH_3$ entirely disappears. On evaporating the solution of albumin, freed from the baryta, a series of crystalline compounds were obtained, to two of which I will now call attention. He says, 'I have found at least two acids' [in the solution of hydrolysed albumin] 'which by their presence prevent the complete precipitation of the baryta by means of carbonic acid, from the solution. They are either crystallisable with difficulty or not at all, are deliquescent and cannot be precipitated by nitrate of mercury. These characters make their examination and their separation extremely difficult. One of them has the composition of an isomeride of aspartic acid ($C_4H_7NO_4$), already found by Kessler in the products of the decomposition of albumin by sulphuric acid, but differs from aspartic acid by being highly soluble in water.

Another found in small quantities, gives numbers corresponding to the formula of a di-amido-citric acid.'

Unfortunately, in neither of these cases does Schutzenberger give his analyses, so that with regard to the latter substance we are left in doubt. The first substance, the isomeride of aspartic acid, is, I suggest, iso-aspartic acid or amino-iso-succinic acid

$$CH_3{\cdot}C(NH_2){\cdot}(COOH)_2 = C_4H_7NO_4$$

derived from the proteid constituent diacetyl-dicyanide and NH_3, or from the combination of methyl-tartronic nitrile with NH_3 and its subsequent saponification.

The other compound 'corresponding to the formula of a di-amido citric acid' is, I suggest, $C_6H_{12}N_2O_6$, differing from diamido-citric acid $C_6H_{11}N_2O_6$ by one atom of hydrogen, and if so, it is a compound of glycocoll with the above-mentioned iso-aspartic acid

$$C_6H_{12}N_2O_6 = \underset{\text{glycocoll}}{CH_2{\cdot}(NH_2){\cdot}COOH} + \underset{\text{iso-aspartic acid}}{CH_3{\cdot}C(NH_2){\cdot}(COOH)_2}$$

resulting from the saponification of the proteid constituents amino-malonic nitrile and methyl-tartronic nitrile

$$\underset{\text{amino-malonic nitrile}}{CH(NH_2){\cdot}(CN)_2} + 3H_2O + \underset{\text{methyl-tartronic nitrile}}{CH_3{\cdot}C(OH){\cdot}(CN)_2} + 2H_2O$$

which incompletely hydrolysed with baryta in a sealed tube, give

$$\begin{aligned} & CH_2(NH_2){\cdot}COOH + CO_2 + NH_3 + CH_3{\cdot}C(NH_2){\cdot}(CN)_2 + 2H_2O) \\ & = \underset{\text{glycocoll}}{CH_2(NH_2){\cdot}COOH} + \underset{\text{iso-aspartic acid}}{CH_3{\cdot}C(NH_2){\cdot}(COOH)_2} + CO_2 + 3NH_3 \end{aligned}$$

the CO_2 combining with the baryta and the NH_3 with other constituents of the proteid.

ON THE COMPLETE HYDROLYTIC DECOMPOSITION OF EGG-ALBUMIN AT 180° C.

By P. W. LATHAM, M.D., *Downing Professor of Medicine in the University of Cambridge* (1874-1894).

(*Received April 26th*, 1908)

The fact that *a* lactic acid and CO_2 should originate in the manner I have described in the previous paper[1] is highly suggestive. If these two substances are evolved from muscle during its contraction and have for their antecedent a compound of pyruvic acid or its nitrile with prussic acid, the question at once presents itself—Can we, by combining pyruvic acid and its homologues, or their nitriles, with other substances produce a series of compounds of the same nature and constitution as are obtained by various processes from protein matter; can we, in fact, in this way synthesise a number of, or even all, the protein derivatives?

The task I have set before me is to prove that an affirmative answer must be given to the question; and I propose to do so by shewing that the synthesis of a very large number of compounds which are obtained from protein, or albuminous bodies, including among them Schutzenberger's leucéins (which I shall endeavour to prove are the imino-ketonic acids) and tyroleucine, as well as various pyridine and chinoline derivatives, can be effected by combining pyruvic acid and its homologues with prussic acid, with ammonia or with other substances, such as the fatty aldehydes, and then acting on these combinations in various ways.

I shall then be able to determine the composition of certain compounds or combinations of the above mentioned bodies, which,

1. See pp. 194 and 201.

according to Schutzenberger[1], result from the hydrolysis of albumin, viz. :—

$$C_{27}H_{48}N_6O_{18} \text{ or } 3\,C_9H_{16}N_2O_6 \text{ acides amides fort}$$

$$C_{27}H_{60}N_6O_{12} \text{ or } 3\,C_9H_{20}N_2O_4 \text{ leucines}$$

$$C_{14}H_{28}N_4O_8 \text{ or } 2\,C_7H_{14}N_2O_4 \text{ gluco-proteins}$$

$$\text{and } C_{144}H_{288}N_{32}O_{64} \text{ or } 16\,C_9H_{18}N_2O_4 \text{ gluco-proteins.}$$

The synthesis of tyrosine from oxy- or amido-benzaldehyde will then be discussed; and subsequently the synthesis of Schutzenberger's leucines, that is, of amino-caproic, -valeric, -butyric, -propionic and amino-acetic acids from the various aldehydes; and lastly the synthesis of the aspartic acid series from a similar source.

With the knowledge of the composition of these bodies we shall be able to determine precisely and completely what substances are furnished by the hydrolysis of albumin, as well as their respective amounts. Moreover, knowing the constitution and synthesis of each product we shall then, as I shall endeavour to show, be within measurable distance of determining the constitution and arriving at the synthesis of albumin itself.

On the Synthesis of Pyruvic Acid, and on some Protein Derivatives which can be Synthesised from it

Synthesis of Pyruvic Acid ($CH_3 \cdot CO \cdot COOH$).—This acid may be obtained by carefully acting upon acetyl-cyanide with HCl[2] and heating.

$$\underset{\text{acetyl-cyanide}}{CH_3 \cdot CO \cdot CN} + HCl + 2\,H_2O = \underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH} + NH_4Cl$$

This method is applicable generally for the formation of the *a* ketonic acids from their nitriles. In the cold the amide is first formed.

The acid may also be regarded as a condensation product of

1. *Annales de Chemie et de Physique*, 5me sér., t. XVI, p. 398.
2. Claisen, *Shadwell*, Ber. XI, 620 u. 1563.

oxalic acid and acetic acid, and may be prepared in the following manner :—

To one molecule of di-ethyl-oxalate dissolved in four parts of absolute ether add one molecule of sodium, and then in small quantities at a time a little more than one molecule of acetic ether, taking care that the temperature of the mixture does not rise above the boiling point of the ether. The sodium is gradually dissolved, hydrogen being evolved, and the greater part of the somewhat dark coloured fluid solidifies, after a few hours, into a yellowish crystalline semi-solid mass, which, when drained from the liquid portion, is washed with ether. In this way the almost pure sodium compound of oxalacetic ether is obtained, as a thick white mass.[1]

$$\underset{\text{diethyl oxalate}}{C_2O_4(C_2H_5)_2} + Na + \underset{\text{acetic ether}}{CH_3 \cdot CO_2 \cdot C_2H_5}$$
$$= \underset{\text{sodium oxalacetic ester}}{CO_2 \cdot C_2H_5CO \cdot CHNa \cdot CO_2 \cdot C_2H_5} + C_2H_5 \cdot OH + H$$

On adding dilute H_2SO_4 (10 per cent.) to the ester mixed with H_2O and ether, the sodium salt is resolved into

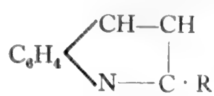
$$\underset{\text{oxalacetic ester}}{CO_2 \cdot C_2H_5 \cdot CO \cdot CH_2 \cdot CO_2 \cdot C_2H_5}$$

which warmed with dilute H_2SO_4 is converted into

$$\underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH}, \ CO_2 \ \text{and} \ \underset{\text{alcohol}}{2\,C_2H_5 \cdot OH}.$$

Some Protein Derivatives the Synthesis of which can be Effected from Pyruvic Acid

The following substances have been obtained from protein :—

Leucéine (Schutzenberger)	$C_3H_5NO_2$
Gluco-protein	$C_6H_{12}N_2O_4$
Alanine or *a* amino-propionic acid	$CH_3 \cdot CH(NH_2) \cdot COOH$
Picoline or methyl-pyridine	$CH_3 \cdot C_5H_4N$
Pyridine	C_5H_5N
Indol	C_8H_7N
Chinoline carboxylates	
and *a* R. Chinolines	

1. Wislicenus, *Annal. d. Chemie*, Bd. 246, s. 315-327.

I will now describe the methods by which, starting with pyruvic acid, these substances can be synthesised.

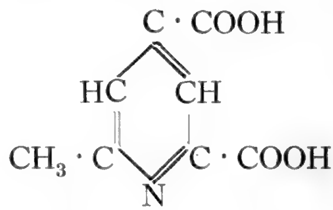
On the Synthesis of Schutzenberger's Leucéine ($C_3H_5NO_2$).—This, I suggest, is imino-pyruvic acid, $CH_3 \cdot C(NH) \cdot COOH$; the ammonium salt of which is precipitated when to a solution of pyruvic acid in ether or alcohol, alcoholic ammonia is added to exact neutralisation.[1]

$$\underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH_3} + 2\,NH_3 = \underset{\text{ammonium imino-pyruvate}}{CH_3 \cdot C(NH) \cdot COONH_4}$$

The ammonium salt is very soluble in water, and reduces Fehling's solution. On boiling it with water it is rapidly decomposed into CO_2, NH_3, uvitaminic acid ($C_9H_{13}NO_7$), and uvitoninic acid ($C_8H_7NO_4$) or picoline dicarboxylic acid, ($CH_3 \cdot C_5H_2N(COOH)_2$)[2]

$$\underset{\text{ammonium imino-pyruvate}}{6\,CH_3 \cdot C(NH) \cdot COONH_4} + H_2O$$

$$= CO_2 + 10\,NH_3 + \underset{\text{uvitaminic acid}}{C_9H_{13}NO_7} +$$



o-picoline . *op*-dicarboxylic acid.

Synthesis of a Alanine ($CH_3 \cdot CH(NH_2) \cdot COOH$).—I have already described the method by which this substance can be synthesised from pyruvic nitrile (see page 203). The synthesis can also be effected directly from pyruvic acid by the following process :—

Take equal molecules of pyruvic acid and concentrated prussic acid and heat them in a sealed tube to 30°-40° C., then add two molecules of alcoholic ammonia and heat the whole to 70° C. Wash the resulting crystals of amino-iso-succinamide with alcohol and then dissolve them in water and recrystallise.[3]

The following are the reactions :—

$$\underset{\text{pyruvic acid}}{CH_3 \cdot CO \cdot COOH} + HCN \rightarrow CH_3 \cdot C(OH \cdot CN)COOH$$

$$CH_3 \cdot C(OH \cdot CN)COOH + 2\,NH_3 \rightarrow \underset{\text{amino-iso-succinamide}}{CH_3 \cdot C(NH_2)\,(CO \cdot NH_2)_2} + H_2O$$

1. Beilstein's *Handbuch d. org. Chemie*, 3te Auf., Bd. 1, s. 587.
2. Bottinger, *Liebig's Annal.*, Bd. 188, s. 330; Bd. 208, s. 138.
3. Körner, Menozzi, *Gazetta chimica Italiana*, Vol. XVII, p. 426.

The crystals appear as large rhombic tablets which are soluble in twenty parts of cold, or three parts of boiling water, but are only slightly soluble in alcohol.

On boiling the amide with hydrochloric acid it is decomposed into CO_2, NH_3 and alanine.

$$\underset{\text{amino-iso-succinamide}}{CH_3 \cdot C(NH_2) \cdot (CONH_2)_2} + 2\,H_2O \rightarrow CO_2 + 2\,NH_3 + \underset{\alpha\ \text{alanine}}{CH_3 \cdot CH(NH_2) \cdot COOH}$$

On the other hand by boiling the amide with one molecule of caustic baryta or other alkali until ammonia is no longer evolved we obtain barium amino-iso-succinate or barium iso-aspartate.[1]

$$CH_3 \cdot C(NH_2) \cdot (CONH_2)_2 + BaH_2O_2 \rightarrow 2\,NH_3 + \underset{\text{barium iso-aspartate}}{CH_3 \cdot C(NH_2) \cdot (COO)_2Ba}$$

On treating the barium salt with an exact equivalent of H_2SO_4 to precipitate the baryta, and then evaporating the filtered solution *in vacuo* over H_2SO_4 we obtain prismatic crystals of iso-aspartic or amino-iso-succinic acid, $CH_3 \cdot C(NH_2) \cdot (COOH)_2$.

On boiling this acid in water it is resolved into CO_2 and α alanine

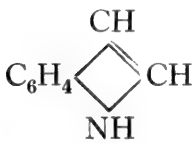
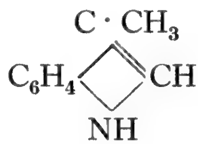
$$\underset{\text{iso-aspartic acid}}{CH_3 \cdot C(NH_2)\,(COOH)_2} = CO_2 + \underset{\alpha\ \text{alanine}}{CH_3 \cdot CH(NH_2)COOH}$$

On the Synthesis of Schutzenberger's Gluco-protein ($C_6H_{12}N_2O_4$) :— Dissolve one molecular equivalent of α alanine in a small quantity of water, then add to it one molecular equivalent of alcoholic ammonia. To one molecular equivalent of pyruvic acid dissolved in alcohol add, very slowly, two molecular equivalents of alcoholic ammonia avoiding a rise of temperature, when the ammonium imino-pyruvate will be formed. Pour into this the solution of alanine, adding, if necessary, a little water to form a clear solution. Evaporate *in vacuo*, when crystalline nodules will be formed consisting of small prisms radiating from a centre the composition of which after crystallisation is a compound of equal molecules of $CH_3 \cdot CH(NH_2) \cdot COOH$ (alanine) and $CH_3 \cdot C(NH) \cdot COOH$ (imino-pyruvic acid), or $C_6H_{12}N_2O_4$, the basic NH_3 having been dissipated.

1. Körner, Menozzi, *loc. cit.*, p. 429.

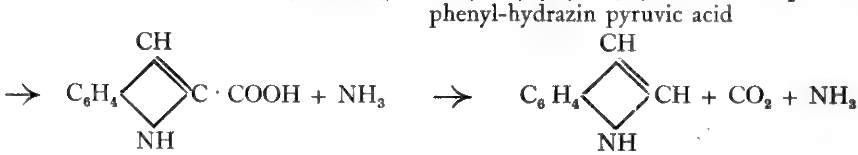
The analysis of the crystals by Mr. M. M. Pattison Muir, who kindly examined them for me, gave the following results :—

Experiment				Calculated for $C_6H_{12}N_2O_4$
C = 40·46	...	...	...	40·91
H = 7·26	...	...	...	6·82
N = 15·6	...	...	...	15·9
O = 36·68	...	...	...	36·37
100·00				100·00

Indol  and skatol  were found by Nenki (Ber. VII (2), 1593; VIII, 336; and X, 1032) and Kühne (Ber. VIII, 206) among the products of putrefying albumin.

Synthesis of Indol.[1]—By adding pyruvic acid to a weak solution of phenyl-hydrazin in acetic acid, phenyl-hydrazin pyruvic acid is precipitated in a crystalline form. This is converted into the ester ; the action of zinc chloride on which in an oil bath at 195° C. is so violent that only small portions at a time should be experimented with. Indol carboxylic acid and its ester are formed. By heating indol carboxylic acid to 230° it is decomposed into CO_2 and indol.

$$CH_3 \cdot CO \cdot COOH + C_6H_5(N_2H_3) = \underset{\text{phenyl-hydrazin pyruvic acid}}{CH_3 \cdot C(C_6H_5 \cdot N_2H)\,COOH} + H_2O$$



indol carboxylic acid — indol

Synthesis of the Chinoline Carboxylates and the a R. Chinolines from Pyruvic Acid.—The chinoline carboxylates $C_nH_{2n-13}NO_2$, containing an alcohol radicle in the Py 2 . position, are formed by heating pyruvic acid with the aldehydes and aniline in alcohol for four to five hours in a water bath ;[2] the aniline being very slowly added during the process.

1. E. Fischer, *Annalen d. Chem.*, Bd. 236, s. 142.
2. Döbner, *Annalen d. Chemie*, Bd. 242, s. 270.

$$CH_3 \cdot CO \cdot COOH + R \cdot CHO + C_6H_5 \cdot NH_2$$
pyruvic acid aldehyde aniline

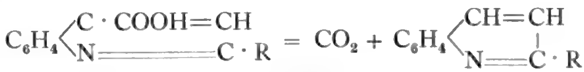
=  $+ 2\,H_2O + H_2$

a R. chinoline carboxylic acid

If the experiment is made at the ordinary temperature neutral bodies are formed

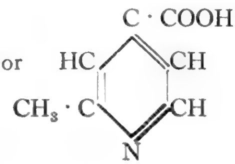
$$R \cdot CHO + CH_3 \cdot CO \cdot COOH + 2\,C_6H_5 \cdot NH_2 = R \cdot CH : C\left\langle \begin{matrix} CH : N \cdot C_6H_5 \\ CO \cdot NH \cdot C_6H_5 \end{matrix} \right.$$

By heating the carboxylates, either by themselves or with soda-lime, they are resolved into *a* R. chinolines and CO_2



Synthesis of Picoline ($CH_3 \cdot C_5H_4N$).—On heating picoline dicarboxylic acid (obtained as above) to 274° C. it is resolved into CO_2 and mono-carboxylic acid[1]

$$\underset{\text{picoline dicarboxylic acid}}{CH_3 \cdot C_5H_2N(COOH)_2} = CO_2 + \underset{\text{picoline carboxylic acid}}{CH_3 \cdot C_5H_3N \cdot COOH}$$ or



2-methyl pyridine -4-carboxylic acid

On fusing the calcium mono-carboxylate with soda-lime, it is converted into CO_2 and picoline[2]

$$\underset{\text{picoline carboxylic acid}}{CH_3 \cdot C_5H_3N \cdot CO_2H} + CaH_2O_2 = \underset{\text{2-picoline or methyl pyridine}}{CH_3 \cdot C_5H_4N} + CaCO_3 + H_2O$$

Synthesis of Pyridine (C_5H_5N).—By oxidising 2-picoline with $KMnO_4$ it is converted into pyridine carboxylic acid[3] :—

$$\underset{\text{picoline}}{CH_3 \cdot C_5H_4N} + O_3 \rightarrow \underset{\text{pyridine carboxylic acid}}{C_5H_4N \cdot COOH} + H_2O$$

1. Bottinger, *Berichte*, Bd. 14, s. 67; Bd. 17, s. 92.
2. Beilstein's *Handb. d. org. Chemie*, Bd. 4, s. 166.
3. Weidel, *Berichte*, Bd. 12, s. 1992.

On heating this with alcoholic potash to 240° C. it is completely transformed into pyridine[1]

$$\underset{\text{pyridine carboxylate}}{C_5H_4N \cdot COOH} + KHO \rightarrow \underset{\text{pyridine}}{C_5H_5N} + KHCO_3$$

Synthesis of Pyrrol ($C_4H_4(NH)$).—On combining uvitaminic acid $C_9H_{13}NO_7$, obtained as above from ammonium imino-pyruvate, with baryta and subjecting the dry salt to the action of heat, a large amount of pyrrol distils over, together with NH_3 and other bases.[2]

On the Synthesis of Acetyl-Acetic Acid and of Certain Protein Derivatives which can be Formed from it

The next higher homologues in the pyruvic acid series are propionyl-formic acid, $CH_3 \cdot CH_2 \cdot CO \cdot COOH$, and acetyl-acetic acid, $CH_3 \cdot CO \cdot CH_2 \cdot COOH$. It is to the products derived from the latter that I will, at present, direct attention. This acid exists in the animal economy, being found in the urine of individuals suffering from diabetes, and is then generally accompanied by β oxybutyric acid, $CH_3 \cdot CH(OH) \cdot CH_2 \cdot COOH$, and acetone $CO \cdot (CH_3)_2$. I suggest that these substances are derivatives of the albuminous tissues.

Synthesis of Acetyl-Acetic Acid ($CH_3 \cdot CO \cdot CH_2 \cdot COOH$).—By acting upon acetic ether $CH_3 \cdot CO_2 \cdot C_2H_5$ with sodium alcoholate, sodium acetyl-acetic ether is produced.

$$2\ \underset{\text{acetic ether}}{CH_3 \cdot CO_2 \cdot C_2H_5} + NaO \cdot C_2H_5 \rightarrow \underset{\text{sodium acetyl-acetic ether}}{CH_3 \cdot CO \cdot CH(Na)CO_2C_2H_5} + 2\ C_2H_5(HO) \cdot$$

On adding acetic acid to this and distilling at 130° C. acetyl-acetic acid $CH_3 \cdot CO \cdot CH_2 \cdot COOH \cdot$ passes over.[3]

The free acid may also be obtained by mixing together 4·5 parts of the ethylic ester with 2·5 parts of KOH and 80 parts of H_2O letting the mixture stand for twenty-four hours, then acidulating with H_2SO_4, and extracting the acid with ether.[4]

1. Beilstein's *Handb. d. Chemie*, 3te Aufl., Bd. IV, s. 141.
2. Beilstein, *loc. cit.*, Bd. I, s. 587.
3. Geuther, *Zeit. f. Chemie*, 1868, s. 652.
4. Ceresole, Ber. XV, p. 139, 1872.

Practically then, we may regard acetyl-acetic acid as the condensed product of two molecules of acetic acid.

$$2\,CH_3 \cdot COOH = CH_3 \cdot CO \cdot CH_2 \cdot COOH + H_2O.$$

On the Synthesis of Schutzenberger's Leucéine ($C_4H_7NO_2$).—This compound is, I suggest, imino-acetyl-acetic acid $CH_3 \cdot C(NH) \cdot CH_2 \cdot COOH$ known also under the name of amino-crotonic acid $CH_3 \cdot C(NH_2) : CH \cdot COOH$,[1] according to the view which is taken of the constitution of the compound.

The ester of this acid may be prepared by mixing together, at the ordinary temperature, acetyl-acetic ether with strong aqueous ammonia, and shaking the mixture, from time to time, for several days.[2]

$$\underset{\text{acetyl-acetic ester}}{CH_3 \cdot CO \cdot CH_2 \cdot COOC_2H_5} + NH_3 \rightarrow \underset{\text{imino-acetyl-acetic ester}}{CH_3 \cdot C(NH) \cdot CH_2 \cdot COOC_2H_5} + H_2O$$

$$\text{or} \rightarrow \underset{\text{amino-crotonic ester}}{CH_3 \cdot C(NH_2) : CH \cdot COOC_2H_5} + H_2O$$

It may also be prepared by passing dry gaseous ammonia rapidly into pure acetyl-acetic ester;[3] mixed with double its volume of ether. The yield being larger if ammonium nitrate is first mixed with the dry ester.

This imino- or amino-body crystallises in colourless thick monoclinic prisms which are not very soluble in water but readily soluble in ether, benzol and chloroform. Its melting point is 34° C. (Collie), 37° (Conrad). It is resolved into its constituents by dilute HCl, and by NaHO. The composition of this body $C_4H_7NO_2$ merely differs from that of amino-butyric acid $CH_3 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ or $C_4H_9NO_2$, by two atoms of hydrogen.

Synthesis of β Oxy-Butyric Acid (β $CH_3 \cdot CHOH \cdot CH_2 \cdot COOH$). If water is added to an alcoholic solution of imino-acetyl-acetic ester until it is slightly opaque and the mixture then placed in an ice bath

1. Conrad, Epstein, *Berichte*, B. 20, s. 3056.
2. Duisberg, *Liebig's Ann.*, Bd. 213, s. 166.
3. J. Norman Collie, *Liebig's Ann.*, Bd. 226, s. 294-301; and Conrad, Epstein, *Berichte*, Bd. 20, s. 3054.

and treated for three days with sodium amalgam we obtain sodium β oxybutyrate[1]

$$CH_3 \cdot C(NH) \cdot CH_2 \cdot COOC_2H_5 + 2\,Na + 3\,H_2O$$
imino-acetyl-acetic ester

$$\rightarrow CH_3 \cdot CH(OH) \cdot CH_2COONa + NH_3 + NaHO + C_2H_5 \cdot HO$$
sodium β oxybutyrate

the solution of which, if exactly neutralised with HCl yields β oxybutyric acid.

It may also be prepared from ethylic aldehyde by converting this by means of dilute HCl into aldol, and then oxidising the latter with oxide of silver.[2]

$$\underset{\text{ethylic aldehyde}}{2\,CH_3 \cdot CHO} \rightarrow \underset{\text{aldol}}{CH_3 \cdot CH(OH) \cdot CH_2 \cdot CHO}$$

$$\text{and } \underset{\text{aldol}}{CH_3 \cdot CH(OH) \cdot CH_2 \cdot CHO} + O \rightarrow \underset{\beta \text{ oxybutyric acid}}{CH_3 \cdot CH(OH) \cdot CH_2COOH}$$

The Synthesis of Acetone ($CO \cdot (CH_3)_2$).—By heating imido-acetyl-acetic ester with dilute NaHO it is resolved into NH_3, CO_2, alcohol and acetone.

$$\underset{\text{imido-acetyl-acetic ester}}{CH_3 \cdot C(NH) \cdot CH_2 \cdot COOC_2H_5} + 2\,NaHO = NH_3 + Na_2CO_3 + \underset{\text{alcohol}}{C_2H_5 \cdot HO} + \underset{\text{acetone}}{CO(CH_3)_2}$$

Acetone may also be obtained from aceto-acetic acid by heating it to a temperature below 100° C., when violent action takes place and it is resolved into CO_2 and acetone.[3]

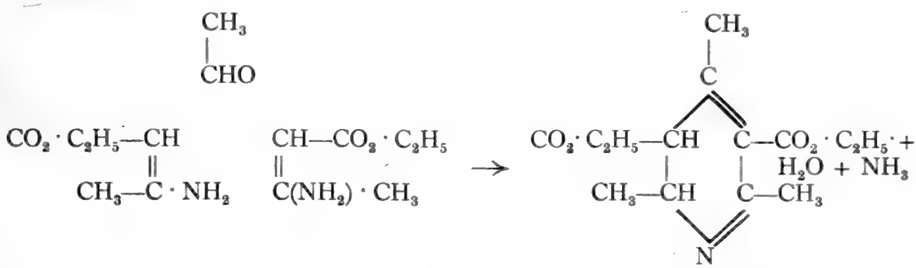
$$\underset{\text{aceto-acetic acid}}{CH_3 \cdot CO \cdot CH_2 \cdot COOH} \rightarrow CO_2 + \underset{\text{acetone}}{CO(CH_3)_2}$$

Synthesis of Collidine from Acetyl-Acetic Ester.—This is effected in the following stages :—

(i) *Synthesis of Hydrocollidine Dicarboxyllic Ester* ($C_5H_2N(CH_3)_3 \cdot (COOC_2H_5)_2$).—This compound may be obtained by warming 52 parts of acetyl-acetic ester with 13·5 parts of aldehyde ammonia ;[4] or by

1. Wislicenus, *Liebig's Ann.*, Bd. 149, s. 205.
2. Beilstein's *Handbuch d. Chemie*, 3te Aufl., Bd. I, s. 1206.
3. *Ibid.*, s. 591.
4. Hantsch, *Liebig's Ann.*, Bd. 215, s. 8.

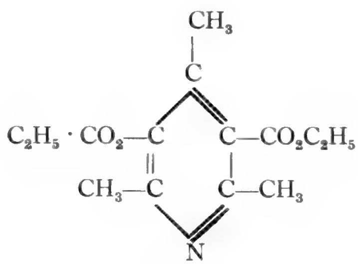
combining β amino-crotonic ester, $CH_3 \cdot C(NH_2) : CH \cdot CO_2C_2H_5$, with paraldehyde and a little H_2SO_4.[1] In the latter case $(CH_3 \cdot CHO)_3$ we have



$\rightarrow C_5H_2N(CH_3)_3(CO_2 \cdot C_2H_5)_2 + H_2O + NH_3$
hydro-collidine-dicarboxyllic ester

(ii) On treating this ester with nitrous acid $H \cdot NO_2$, it loses H_2 and is converted into

$C_5N(CH_3)_3(CO_2C_2H_5)_2$ or



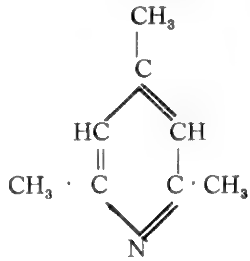
collidine dicarboxyllic-diethyl ester

(iii) From this the mono-ethyl ester can be obtained by heating it with a molecular equivalent of alcoholic potash for some time in a flask, to which a reversible condenser is attached. The solution is then freed from alcohol by distillation and the residue dissolved in water, any unchanged diethyl ester is then extracted with ether or benzol, and the solution neutralised by adding a molecular equivalent of HCl. The solution is then evaporated to dryness in a water bath and the ester recrystallised from absolute alcohol.[2] The result is

$$CO_2H \cdot C_5N(CH_3)_3CO_2 \cdot C_2H_5$$
collidine dicarboxyllic mono-ethyl ester.

1. Collie, *Liebig's Ann.*, Bd. 226, s. 314.
2. R. Michael, *Lieb. Ann.*, Bd. 225, s. 124.

If two molecules of alcoholic potash are employed CO_2 is disengaged and γ collidine or trimethyl-pyridine is left behind.



2.4.6. trimethyl pyridine
or γ collidine.

If the mono-ethyl ester prepared as above is heated to 255° C. it is resolved into CO_2 and

$$C_5NH(CH_3)_3 \cdot CO_2 \cdot C_2H_5$$

collidine carboxylic ester

which distils over.[1]

On saponifying this with alcoholic potash and then adding sufficient HCl to convert the whole of the potash into KCl, evaporating the solution to dryness and treating the residue with absolute alcohol, on evaporation we obtain short needles of

$$C_5NH(CH_3)_3 \cdot CO_2H + 2\ H_2O$$

collidine carboxylic
acid[2]

Synthesis of Lutidine (C_7H_9N).—By the oxidation of one molecule of potassium collidine carboxylate with 2, 4, and 6 molecules of $KMnO_4$ we obtain respectively[3] the potassium salts of

$$C_5NH(CH_3)_2(COOH)_2$$

lutidine dicarboxylic acid

$$C_5NH(CH_3)(COOH)_3$$

picoline tri-carboxylic acid

and $C_5NH \cdot (COOH)_4$

pyridine tetra-carboxylic acid

1. *Ibid.*, s. 131.
2. *Ibid.*, s. 134.
3. *Ibid.*, s. 136.

By fusing potassium lutidine dicarboxylate with lime we obtain[1]

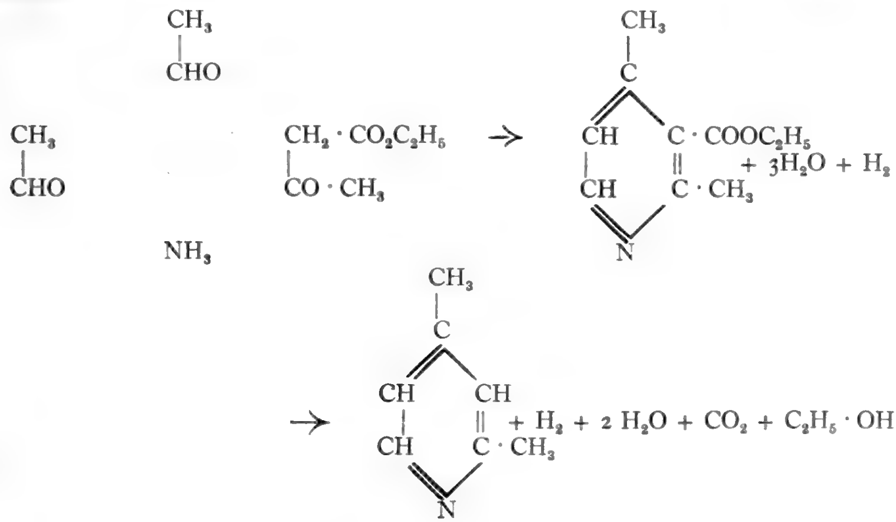
$$C_5H_3N(CH_3)_2$$

lutidine
or dimethyl pyridine

β Lutidine can also be obtained by mixing together 130 grammes aceto-acetic ester, 61 grammes aldehyde ammonia and 50 grammes of aldehyde[2] at 130° C., to form lutidine carboxylic ester.

$$CH_3 \cdot CO \cdot CH_2 \cdot CO_2 \cdot C_2H_5 + CH_3 \cdot CHO \cdot NH_3 + CH_3 \cdot CHO$$
$$= (CH_3)_2 \cdot C_5H_2N \cdot CO_2C_2H_5 + 3\ H_2O + H_2.$$

From this lutidine carboxylate can be obtained, which fused with lime is resolved into CO_2 and 2·4 dimethyl pyridine or lutidine.



2·4 dimethyl pyridine
or lutidine.

On the Synthesis of Tyroleucine ($C_7H_{11}NO_2$)

Perhaps the most important of Schutzenberger's experiments as elucidating the constitution of the leucéins is the one by which he succeeded in isolating tyroleucine $C_7H_{11}NO_2$ and caproic leucéine $C_6H_{11}NO_2$.

1. Engelmann, *Lieb. Ann.*, Bd. 231, s. 54.
2. Michael, *Ber.*, Bd. 18, s. 2022.

The following are the details of his experiment[1] :—

'Ten kilogrammes of albumen were decomposed by baryta in a large autoclave, and the liquid having been freed successively from ammonia and from the excess of baryta by a current of carbonic acid and by sulphuric acid, it was then concentrated till it crystallised. I was able to isolate about 2 kilogrammes of crystalline deposit corresponding to the deposit (*A*).

'From this considerable mass I was only able to procure—by repeated crystallisations carefully carried as far as possible—first, the substances mentioned already, tyrosine, leucine, butalanine, amido-butyric acid, crystalline compounds of the type $C_mH_{2m}N_2O_4$ (m = 12 and 10), and secondly, two new definite and crystallisable products, of which one, to which I give the name *Tyroleucine*, corresponds to the formula $C_7H_{11}NO_2$, and the other belonging to the type $C_nH_{2n-1}NO_2$; its composition is represented by the formula $C_6H_{11}NO_2$; it is a leucéine (n = 6), caproic leucéine. Tyroleucine belongs to the series $C_nH_{2n-3}NO_2$; it plays only a very secondary part in the composition of albumen, and appears in small quantities only in the products of its decomposition. With 100 grammes of substance, I completely overlooked its presence. By experimenting with 10 kilogrammes I was able to obtain from 60 to 70 grammes in a pure state. The existence of a definite and crystallised substance belonging to the type $C_nH_{2n-1}NO_2$ tends to prove that the compounds of the type $C_mH_{2m}N_2O_4$, which are so abundant, should be regarded as molecular combinations of leucines and of leucéines.

'The following experiment, however, seems to shew that the compounds $C_mH_{2m}N_2O_4$ (m = 10 or 12) cannot always be separated, by successive crystallisations into leucines $C_nH_{2n+1}NO_2$ and leucéines $C_nH_{2n-1}NO_2$. A crystalline deposit in nodules or spherical masses radiating from a centre, gave the following result.

(*a*)	Substance	0·3495
	Carbonic acid ...	0·707
	Water	0·286

1. *Annales de Chimie et de Physique*, 5me série, t. XVI, pp. 345, 352.

'These crystals, re-dissolved in water and purified by a fresh crystallisation, gave

(*b*)	Substance	... ...	0·3105
	Carbonic acid	...	0·631
	Water	... ...	0·251

The percentage being			Calculated for
	a	*b*	$C_{12}H_{24}N_2O_4$
Carbon	55·1	55·42	55·38
Hydrogen	9·09	9·01	9·2

The composition remained unaltered, and we must therefore admit the existence of a body corresponding to the formula $C_{12}H_{24}N_2O_4$ ($m = 12$). This result has been frequently verified, not only as to the compound $C_{12}H_{24}N_2O_4$, but also for the lower homologues $C_{11}H_{22}N_2O_4$, $C_{10}H_{20}N_2O_4$, $C_9H_{18}N_2O_4$. In the experiment with 10 kilos of albumen, tyroleucine ($C_7H_{11}NO_2$) and caproic leucéine ($C_6H_{11}NO_2$) were extracted from the aqueous solution of the crystalline deposit (*A*) by first separating the greater part of the tyrosine and leucine, by means of fractional crystallisations. The mother-liquor, freed from traces of baryta by sulphuric acid, was decolourised by animal charcoal, and then again concentrated. A fairly copious deposit of spherical masses took place. From this I was able to obtain a considerable amount of two new bodies. This deposit was again treated with hot water, which dissolved it all except a residue of tyrosine; the dark liquid was decolourised by means of a little sub-acetate of lead, and filtered from the brown flocculi; the excess of lead was precipitated by sulphuretted hydrogen and the liquid filtered and decolourised by animal charcoal. This was twice concentrated and two crystalline products (*B*) and (*C*) in spherical masses or in granules were obtained.

'The crystals (*B*) purified and recrystallised were composed of tyroleucine $C_7H_{11}NO_2$.

'At a temperature of from 250° to 280° in an inert gaseous atmosphere tyroleucine is decomposed, furnishing a white sublimate, water, and carbonic acid, together with an oily volatile base having

the odour of horse-radish, which remains in the retort as an amorphous, transparent mass having a resinous fracture.

'An analysis of the chloroplatinate of the oily base shewed its composition to be $C_8H_{11}N$ which represents collidine or an isomeric substance. The vitreous residue on analysis furnished numbers corresponding with the formula $C_{14}H_{18}N_2O_2$. It appears from these results that tyroleucine heated above 250° is in part dehydrated and polymerised, while another portion is decomposed into carbonic acid, collidine and butalanine in accordance with the following equation :—

$$2\ C_7H_{11}NO_2 = CO_2 + C_8H_{11}N + C_5H_{11}NO_2$$

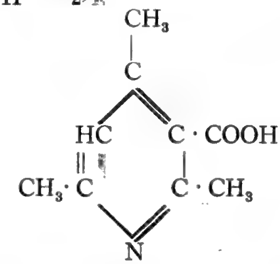
Dehydration takes place as in the following equation :—

$$2\ C_7H_{11}NO_2 = 2\ H_2O + C_{14}H_{18}N_2O_2$$

Tyroleucine, therefore, must be regarded as a compound of butalanine (amino-valerianic acid) with a body whose composition is $C_9H_{11}NO_2$, differing from tyrosine by one atom of oxygen.

'An analysis of the crystals (*C*), obtained by concentrating the mother-liquor from which the tyroleucine had been obtained, shewed that it consisted of $C_6H_{11}NO_2$ or leucéine caproic.'

From the above extract it is evident that tyroleucine is composed of amino-valerianic acid $CH_3 \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH$ and collidine carboxylate $C_9H_{11}NO_2$, or



which is obtained as shown on page 218 by heating collidine dicarboxylate, a compound which results from oxidation of hydrocollidine dicarboxylic ester, the latter being obtained by combining paraldehyde with the ester of β amino-crotonic acid.[1]

$$CH_3 \cdot CHO + 2\ CH_3 \cdot C(NH_2) : CH \cdot COOC_2H_5$$
$$= C_5H_2N(CH_3)_3(CO_2 \cdot C_2H_5)_2 + H_2O + NH_3$$

1. Collie, *Liebig's Ann.*, Bd. 226, s. 314.

Higher Homologues of the Pyruvic Acid Series

The next higher homologues of the pyruvic series are—Methyl-aceto-acetic acid $CH_3 \cdot CO \cdot CH(CH_3) \cdot COOH$, and ethyl-aceto-acetic acid $CH_3 \cdot CO \cdot CH(C_2H_5) \cdot COOH$ and two others isomeric with them, namely, β acetyl-propionic acid or laevulinic acid $CH_2 \cdot CO \cdot CH_2 \cdot CH_2 \cdot COOH$, and γ acetyl - butyric acid $CH_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$.

On the Synthesis of Methyl-Aceto-Acetic Acid and Ethyl-Aceto-Acetic Acid.—These two bodies are obtained by the action of the iodides of methyl and ethyl respectively on sodium acetyl-acetic ester. The reaction takes place most readily by dissolving the theoretical amount of sodium in ten to twelve times the weight of absolute alcohol and, after the mixture has cooled, adding the aceto-acetic ester and immediately afterwards iodide of methyl or ethyl (as the case may be) until the reaction is neutral. Distil off the greater portion of the alcohol, and then add sufficient water to the residue to dissolve the whole of the sodium salt.[1]

The following are the reactions :—

$$\underset{\text{aceto-acetic ester}}{CH_3 \cdot CO \cdot CH_2 \cdot CO_2 \cdot C_2H_5} + NaO \cdot C_2H_5 \rightarrow \underset{\text{sodium aceto-acetic ester}}{CH_3 \cdot CO \cdot CH(Na)CO_2 \cdot C_2H_5} + C_2H_5(OH)$$

$$\underset{\text{sodium aceto-acetic ester}}{CH_3 \cdot CO \cdot CH(Na)CO_2 \cdot C_2H_5} + CH \cdot I \rightarrow \underset{\text{methyl aceto-acetic ester}}{CH_3 \cdot CO \cdot CH(CH_3)CO_2 \cdot C_2H_5} + NaI$$

and—

$$\underset{\text{sodium aceto-acetic ester}}{CH_3 \cdot CO \cdot CH(Na)CO_2 \cdot C_2H_5} + C_2H_5 \cdot I \rightarrow \underset{\text{ethyl aceto-acetic ester}}{CH_3 \cdot CO \cdot CH(C_2H_5)CO_2 \cdot C_2H_5} + NaI$$

These two esters if acted upon by 2KHO are resolved into $K_2CO_3 + HO \cdot C_2H_5$ and $CH_3 \cdot CO \cdot CH_2 \cdot CH_3$ (methyl-ethyl ketone) and $CH_3 \cdot CO \cdot CH_2 \cdot C_2H_5$ (methyl-propyl ketone) respectively.

The two other homologues of pyruvic acid, isomeric respectively with methyl-acetyl-acetic acid and ethyl-acetyl-acetic acid, viz.,

1. Conrad, Limpach, *Lieb. Ann.*, Bd. 192, s. 153.

laevulinic acid or β acetyl-propionic acid ($CH_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot COOH$) and γ acetyl-butyric acid ($CH_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$), may also be obtained from sodium diacetic ester.

Synthesis of Imino-Laevulinic Acid.—By acting upon sodium-diacetic-ethyl ester with chlor-acetic ester, acetyl-succinic diethyl ester is produced.[1]

$$\underset{\text{sodium diacetic ester}}{CH_3 \cdot CO \cdot CHNa \cdot CO_2 \cdot C_2H_5} + \underset{\text{chloracetic ester}}{CH_2Cl \cdot CO_2C_2H_5}$$

$$\rightarrow NaCl + CH_3 \cdot CO \cdot CH(CH_2 \cdot CO_2 \cdot C_2H_5)\, CO_2 \cdot C_2H_5$$

$$\rightarrow NaCl + \underset{\text{acetyl-succinic diethyl ester}}{C_2H_5 - CO_2 \cdot CH(CO \cdot CH_3)CH_2 \cdot CO_2 \cdot C_2H_5}$$

Rash[2] prepared this by taking 52 grammes of acetyl-acetic ester, 9·2 grammes Na, 150 grammes $C_2H_5 \cdot OH$, and 125 grammes chlor-acetic ester.

On boiling the acetyl-succinic ester with twice its volume of dilute HCl, or with baryta water, it is resolved into CO_2, $C_2H_5 \cdot OH$ and $CH_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot COOH$ (β acetyl-propionic acid) or laevulinic acid, together with a little acetic and succinic acids.

This combines with NH_3 to form $CH_3 \cdot C(NH) \cdot CH_2 \cdot CH_2 \cdot COOH$ (imino-laevulinic acid).

Synthesis of Imino-Acetyl Butyric Acid ($CH_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$).—By acting upon sodium diacetic ester with β iodo-propionic acid, acetyl-glutaric ester is produced.[3]

$$CH_3 \cdot CO \cdot CHNa \cdot CO_2C_2H_5 + CH_2I \cdot CH_2 \cdot CO_2 \cdot C_2H_5$$

$$\rightarrow \underset{\text{acetyl-glutaric ester.}}{\begin{array}{l} \qquad\quad CH_2 \cdot CH_2 \cdot CO_2 \cdot C_2H_5 \\ \qquad\quad | \\ CH_3 \cdot CO \cdot CH \cdot CO_2C_2H_5 \end{array}} + NaI$$

This boiled for eight to ten hours with one part of concentrated HCl and two parts of water is resolved into CO_2, alcohol, and γ acetyl-butyric acid.[4]

$$= CO_2 + 2C_2H_5Cl + \underset{\gamma\text{ acetyl-butyric acid}}{CH_3 \cdot CO \cdot (CH_2)_3 \cdot COOH}$$

1. Conrad, *Annalen*, Bd. 188, s. 218.
2. *Annalen*, B. 234, s. 36.
3. Wislicenus, Limpach, *An. d. Chemie*, p. 192, 128.
4. Wolff, *An. d. Ch.*, p. 216, 129.

Treated with a strong solution of alcoholic potash it is resolved into acetic acid and glutaric acid.

$$= \underset{\text{pot. acetate}}{CH_3 \cdot CO_2K} + \underset{\text{alcohol}}{2C_2H_5 \cdot HO} + \underset{\text{pot. glutarate}}{\begin{array}{l} CH_2 \cdot CH \cdot COOK \\ | \\ CH_2 \cdot COOK \end{array}}$$

Combined with NH_3 acetyl-butyric acid forms $CH_3 \cdot C(NH) \cdot (CH_2)_3 \cdot COOH$ (imino-acetyl butyric acid).

SYNTHESIS OF SCHUTZENBERGER'S LEUCÉINES $C_5H_9NO_2$ and $C_6H_{11}NO_2$

Each of these bodies may be represented by two isomeric compounds. The leucéine $C_5H_9NO_2$ may be represented either by—

$$CH_3 \cdot C(NH_2) : C(CH_3) \cdot COOH$$

β amino-α methyl-crotonic acid

or by $CH_3 \cdot C(NH) \cdot CH_2 \cdot CH_2 \cdot COOH$

imino-laevulinic acid

The leucéine $C_6H_{11}NO_2$ being represented by—

$$CH_3 \cdot C(NH_2) : C(C_2H_5) \cdot COOH$$

β amino-α ethyl-crotonic acid

or by $CH_3 \cdot C(NH) \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$

γ imino-acetyl-butyric acid

Each of these four leucéines being, I suggest, derivatives of protein.

The synthesis of the ester of the two amino-crotonic acids may be effected by passing NH_3 gas into an ethereal solution of methyl-crotonic and ethyl-crotonic ester respectively,[1] as in the formation of β amino-crotonic ester from acetyl-acetic ester.[2]

Or, the two esters may be formed from β amino-crotonic ester by acting upon it first by sodium ethylate and treating the resulting compound with CH_3I and C_2H_5I respectively.

$$CH_3 \cdot C(NH_2) : CH \cdot CO_2C_2H_5 + NaOC_2H_5 \rightarrow$$
$$CH_3 \cdot C(NH_2) : CNa \cdot CO_2C_2H_5 + HO \cdot C_2H_5$$

and

$$CH_3 \cdot C(NH_2) : CNa \cdot CO_2C_2H_5 + CH_3I \rightarrow$$
$$CH_3 \cdot C(NH_2) : C(CH_3)CO_2C_2H_5 + Na\ I$$

β amino-α methyl-crotonic acid

1. Conrad, Epstein, *Berichte*, Bd. 20, s. 3055 u. 3057.
2. See page 215.

Acting on the esters of these two amino-acids with dilute HCl they are converted into the corresponding crotonic esters and NH_4Cl; and these acted upon by KHO are converted into the corresponding ketones.[1]

I therefore venture to submit that these imino compounds of pyruvic acid and its homologues are the compounds which, in combination with the amino-fatty acids, Schutzenberger obtained by the hydrolytic decomposition of albumin—and to which he gave the name of leucéins, the combinations with the amino-fatty acids being named by him gluco-proteins.

Having made this advance we are now in a position to discuss the results which he obtained in a remarkable series of researches extending over several years. These results are published in *Comptes Rendus*, tome 80, p. 233; tome 81, p. 1108; tome 84, p. 124; tome 101, p. 1267; tome 102, p. 1289; tome 106, p. 1407; and tome 112, p. 198. The most important paper is contained in the *Annales de Chimie et de Physique*, 5me série, tome 16, p. 289, and gives the results of innumerable analyses made with extreme accuracy. From this paper I shall take the details which are necessary for my present purpose.

In his researches Schutzenberger employed white of egg coagulated by heat and with a slight excess of acetic acid. This was well washed with water, alcohol and ether—and dried at 140° C. Fifty or one hundred grammes of this mixed with water were treated with two to six times its weight of crystalline barium hydrate in a closed iron vessel, and heated to a temperature ranging from 100° C. to 250° C., for periods varying from eight to one hundred and twenty hours (*loc. cit.*, p. 303). After the vessel had completely cooled down it was opened; generally there was entire absence of increased pressure or of unabsorbed gas. Sometimes if the temperature had been raised to near 200° and if a large proportion of baryta had been used, a certain

1. See page 223.

amount of hydrogen uncontaminated by carbon escaped from the digester. This Schutzenberger attributed to some secondary action of the baryta on the iron vessel.[1]

The contents of the digester were now :—(i) Subjected to distillation and the amount of NH_3 determined. (ii) The insoluble deposit was separated from the liquid portion and the amount of barium carbonate and oxalate in the deposit was determined. (iii) The baryta contained in the filtered liquid was precipitated first as far as possible by passing a current of CO_2 through the solution kept at the boiling point for a considerable time,[2] then filtering off the precipitate and washing it : concentrating the filtrate and the washings and adding the *exact* quantity of H_2SO_4 necessary to precipitate the baryta still held in solution. (iv) The acid filtrate from these precipitates was then distilled and the amount of acetic acid contained in the distillate determined. In addition to acetic acid the distillate contained traces of formic acid, and an essential volatile oil consisting of pyrrol, etc. (v) The liquid was finally evaporated to dryness in a water bath. This dried residue was called by Schutzenberger the 'résidu fixe,' meaning thereby a mixture of substances which do not sublime nor volatilize at a temperature below 100° C.

Having advanced so far, Schutzenberger's next step was to endeavour from the analysis of albumin to construct such a molecular formula as might serve as a working hypothesis. Taking tyrosine as a basis, the percentage of which can be readily determined, as it crystallises readily and is only slightly soluble in cold water, he found as the result of numerous most carefully conducted experiments that the amount obtained from 100 grammes of albumin ranged between 2·5 and 3·5 grammes, the mean result being 3·4. Now it is improbable that tyrosine results from the combination of several molecules of albumin ; consequently, if we assume that one molecule

1. *Annales de Chimie et Physique*, 5me série, t. XVI, p. 296. Later on see page 230. I shall endeavour to shew that this hydrogen resulted from the action of the baryta on formic acid converting it into oxalic acid and hydrogen.

2. *Loc. cit.*, p. 298.

of tyrosine (= 181) is produced by the decomposition of one molecule of albumin, and that this contains 3·3 per cent. of tyrosine, we have as the molecular weight of albumin—

$$\frac{181 \times 100}{3 \cdot 3} = 5484$$

On this ground alone Lieberkühn's formula $C_{72}H_{112}N_{18}O_{22}S$, of which the molecular weight is 1612, cannot be accepted as correct. Moreover, as Schutzenberger points out,[1] the proportion of nitrogen (15·8 per cent. in Lieberkühn's formula) is too small; the actual percentage being 16·6. Schutzenberger's analysis of albumin[2] gives the following results :—

Carbon	52·57
Hydrogen	7·16
Nitrogen	16·6
Oxygen	21·8
Sulphur	1·8
	99·93

This agrees closely with those of

	Brittner[3] and	Fleitman[4]
C ...	54·9	53·8
H ...	7·0	7·3
N ...	16·6	16·2
S ...	1·6	1·4

Based on this analysis he adopts as the formula for albumin, $C_{240}H_{387}N_{65}O_{75}S_3$ having a molecular weight of 5473, and the percentage composition of which is :—

Carbon	52·62
Hydrogen	7·07
Nitrogen	16·62
Oxygen	21·94
Sulphur	1·75
	100·00

1. *Loc. cit.*, p. 383.
2. *Loc. cit.*, p. 384.
3. Beilstein, *Handb. d. Chemie*, 3te Aufl., Bd. IV, s. 1590.
4. *Watt's Dict.*, Vol. IV, p. 738.

The formula $C_{240}H_{397}N_{65}O_{75}S_3$, differing from the above by ten atoms of hydrogen, and having a molecular weight of 5483, corresponds more closely with Schutzenberger's analysis, and is the one I shall adopt. Its percentage composition is :—

Carbon ...	...	...	52·53
Hydrogen	...	...	7·24
Nitrogen	...	...	16·6
Oxygen ...	...	...	21·88
Sulphur ...	...	...	1·75
			100·00

and I will endeavour by its aid to interpret the analytical results which Schutzenberger obtained.

Acting upon 100 grammes of albumin, in the manner previously described (see page 226), at a temperature of 180° C. with four or five parts of baryta he obtained

Nitrogen (in the form of NH_3)		...	...	4·03
Barium oxalate ...	...	...	...	17·6
Barium carbonate ...	...	...	...	11·0
Acetic acid ...	...	...	...	4·6—4·9

which, for a molecular weight of 5483, correspond very closely to

	16	molecules of	Ammonia
	4	,,	Oxalic acid
	3	,,	Carbonic acid
and	4	,,	Acetic acid

The 'résidu fixe' obtained under the same conditions after separating the baryta with CO_2 and then with H_2SO_4 and evaporating to dryness weighed 99·6 grammes, and on analysis yielded the following percentages very approximately[1]

C	=	48·63
H	=	7·97
N	=	12·58
O	=	30·82
		100·00

1. *Loc. cit.*, p. 385.

giving for the 'résidu fixe' the formula $C_{221}H_{435}N_{49}O_{105}$, the calculated percentage of which is—

C	=	48·95
H	=	8·03
N	=	12·01
O	=	31·01
		100·00

Schutzenberger, moreover, determined that the decomposition of albumin by baryta under the conditions specified was one of hydrolysis, and that during the process one molecule of albumin at 180° C. combined with 60 molecules of water. With the results just given, the following equation represents the decomposition :—

$$\underset{\text{albumin}}{C_{240}H_{397}H_{65}N_{75}S_3} + 60\,H_2O = \underset{\text{résidu fixe}}{C_{221}H_{435}N_{49}O_{105}}$$
$$+ \underbrace{(16\,NH_3 + 4(COOH)_2 + 3\,CO_2 + 4\,CH_3 \cdot COOH + S_3)}_{\text{Schutzenberger's polynomial}} + 5H_2$$

the intermediate stage being :—

$$C_{221}H_{435}N_{49}O_{105} + 16\,NH_3 + (COOH)_2 + 6\,H\,(COOH) + 3\,CO_2 + 4\,CH_3 \cdot CO_2H + 2\,SH_2 + S^*$$

We have now to deal exclusively with the 'résidu fixe' $C_{221}H_{435}N_{49}O_{105}$. After trying by various methods to separate the constituents of this compound, Schutzenberger arrived at the conclusion that the only feasible plan was by fractional crystallisation from various neutral solvents such as water, alcohol, ether, etc. 'The different crystalline products can be distinguished, to a certain extent, by their form and appearance, especially when examined under the microscope. But, as we are well aware, the bodies of which the "résidu" is composed belong to small groups, each members of a homologous series, and frequently there is very little difference in the characters of the neighbouring terms of any single group. Moreover, two terms of the same group, or of two neighbouring groups, have a general tendency to crystallise together in their molecular proportions.

* $\underset{\text{formic acid}}{6H \cdot COOH} + 2SH_2 + S$ being under the action of the BaH_2O_2 converted into $\underset{\text{oxalic acid}}{3\,(COOH)_2} + S_3 + 5H_2$

Intermediate products, therefore, are frequently obtained, adding considerably to the difficulty of arriving at a proper estimate.'[1]

The conclusions at which Schutzenberger arrived were based upon the results of upwards of five hundred analyses of the various products which he obtained from the 'résidu fixe.' In order to separate these products he found that in the first instance the best plan was to pass through the primary solution freed from acetic acid and filtered from the barium carbonate and oxalate a stream of carbonic acid for a considerable time, in order to precipitate the excess of baryta, and after filtering from the $BaCO_3$ to concentrate the solution, without precipitating the baryta which it still contains, with sulphuric acid. After a certain degree of concentration a crystalline pellicle forms on the surface and, on cooling, a copious crystalline mass (*A*) separated out, consisting of granules formed by the aggregation of crystals round a centre. Further concentration of the mother-liquor furnished additional crystals. Finally there remained a voluminous syrup (*B*) which required subsequent treatment to obtain its constituents in a crystallised form.

From the crystalline mass (*A*) crystals of tyrosine $C_9H_{11}NO_3$, and leucine $C_6H_{13}NO_2$, together with amino-valerianic acid $C_5H_{11}NO_2$, were obtained. These two latter were combined in various ways with the leucéins $C_6H_{11}NO_2$ and $C_5H_9NO_2$, forming the crystalline compounds—

	$C_{12}H_{24}N_2O_4$	composed of	$C_6H_{13}NO_2 + C_6H_{11}NO_2$
			leucine leucéine
	$C_{11}H_{22}N_2O_4$	either	$C_6H_{13}NO_2 + C_5H_9NO_2$
		or	$C_5H_{11}NO_2 + C_6H_{11}NO_2$
and	$C_{10}H_{20}N_2O_4$	composed of	$C_5H_{11}NO_2 + C_5H_9NO_2$

The last fractional crystallisation from the mass (*A*) on analysis, furnished numbers which agree with those of amino-butyric acid, $C_4H_9NO_2$. Schutzenberger makes no mention here of the presence of amino-propionic acid $C_3H_7NO_2$. From analyses and statements, however, which will be found further on it is evident that it was

1. *Loc. cit.*, p. 332.

present, probably combined with $C_5H_{11}NO_2$ and so forming the compound 2 $C_4H_9NO_2$.

Caproic leucéine, $C_6H_{11}NO_2$, crystallises in granules consisting of needles grouped round a centre, and is more soluble in water than leucine. It is soluble in boiling alcohol, and has a slightly sweet taste.[1]

Amino-valerianic acid $C_5H_{11}NO_2$ is found in the primary crystallisations of tyrosine, tyroleucine, leucine and caproic leucéine, together with the intermediate products $C_mH_{2m}N_2O_4$ (m = 12, 11, and 10). It is more soluble in water than leucine, and is most readily obtained from the mother-liquor after the separation of the above products. It is therein associated with a considerable proportion of amido-butyric acid $C_4H_9NO_2$ and traces of alanine or amino-propionic acid, $C_3H_7NO_2$. This mother-liquor also contains the lower members of the series $C_mH_{2m}N_2O_4$ and $C_nH_{2n+1}NO_2$ (m = 10-7; n = 5-4).[1]

For the extraction of amino-valerianic acid and the other substances referred to above, which are very soluble in water, the following process was adopted :—

The mother-liquor or uncrystallisable syrup (*B*) was first completely deprived of baryta by means of sulphuric acid and then evaporated to dryness. The residue was treated several times with boiling alcohol, which almost entirely dissolved it, and on cooling furnished a crystalline deposit. This deposit weighed about 3 or 4 per cent. of the albumin operated upon, and consisted of $C_9H_{20}N_2O_4$, crystallising from the alcoholic solution in tufts (houppes) ; this is a mixture of amino-butyric acids.

$$C_9H_{20}N_2O_4 = \underbrace{C_5H_{11}NO_2}_{\text{amino-valerianic acid}} + \underbrace{C_4H_9NO_2}_{\text{amino-butyric acid}}.$$

These two acids have a marked tendency to crystallise together in their molecular proportions, especially from an alcoholic solution. A large number of analyses were made of the crystalline deposits from

1. *Loc. cit*, p. 352.

the alcoholic solutions of the dried residue of the mother-liquid (*B*) obtained in the manner above described. These analyses always indicated the presence of a compound of two allied substances. The separation of these substances, however, could be accomplished by using water as a solvent, in the following manner :—After dissolving the compound $C_9H_{20}N_2O_4$ [$= C_5H_{11}NO_2 + C_4H_9NO_2$] in water, the solution was decolourised with animal charcoal and then concentrated *in vacuo*, at a gentle heat (40° to 50° C.). Butalanine crystallised out during ebullition in the form of white leaflets resembling those of caprioic alanine, which, if present, was also deposited. The mother-liquid then, when highly concentrated, yielded crystals of amino-butyric acid.

Amino-valerianic acid closely resembles amino-caproic acid, or leucine, in appearance. It is almost equally volatile and more soluble in water, and under the microscope it appears as crystalline nodules, formed by short needles radiating from a centre and not in flat plates. It has a somewhat sweetish taste.

Amino-butyric acid is a comparatively large ingredient of the 'résidu fixe.' It crystallises, from a hot alcoholic solution, on cooling in small delicate pearly leaves resembling amino-caproic acid. It is almost insoluble in cold, but is more soluble in hot alcohol. It is readily soluble in water from which, when sufficiently concentrated, it crystallises in the form of nodules composed of needles grouped round a centre. It has a sweeter taste than amino-valerianic, or amido-caproic acid.

The extract obtained by boiling absolute alcohol furnished also the following products[1] :—

(1) The leucines $C_5H_{11}NO_2$ (amino-valerianic acid), $C_4H_9NO_2$ (amino-butyric acid), $C_3H_7NO_2$ (amino-propionic acid).

(2) The leucéins $C_5H_9NO_2$, $C_4H_7NO_2$, $C_3H_5NO_2$, and

(3) Their intermediate products or combinations $C_{10}H_{20}N_2O_4$, $C_9H_{18}N_2O_4$, $C_8H_{16}N_2O_4$, $C_7H_{14}N_2O_4$.

1. *Loc. cit.*, pp. 359, 369.

To these products, together with the two higher members of the series $C_{12}H_{24}N_2O_4$ and $C_{11}H_{22}N_2O_4$, Schutzenberger gave the name of gluco-proteins.

After completely exhausting the 'résidu fixe' with boiling absolute alcohol there still remains, as stated on page 231, a certain quantity of matter, amounting to about 3 or 4 per cent. of the albumin, which is readily soluble in water, and has a somewhat sweet taste. The aqueous solution after concentration becomes after a time a mass of crystalline grains. Its analysis corresponds to the formula $C_7H_{14}N_2O_4$.

These, then, are some of the substances (among many others) which, starting with pyruvic acid and its three higher homologues, can be synthesised in the laboratory. Their compounds with NH_3, viz. :—

(i) $CH_3 \cdot C(NH) \cdot COOH$ (imino-pyruvic acid) ; (ii) $CH_3 \cdot C(NH) \cdot CH_2 \cdot COOH$ (imino-acetyl-acetic acid) ; or $CH_3 \cdot C(NH_2) : CH \cdot COOH$ (amino-crotonic acid) ; (iii) $CH_3 \cdot C(NH) \cdot CH(CH_3) \cdot COOH$ (imino-methyl-acetyl-acetic acid) ; or, $CH_3 \cdot C(NH_2) : C(CH_3)\ COOH$ (amino-methyl-crotonic acid) ; and (iv) $CH_3 \cdot C(NH) \cdot CH(C_2H_5) \cdot COOH$ (imino-ethyl-acetyl-acetic acid) ; or, $CH_3 \cdot C(NH_2) : C(C_2H_5) \cdot COOH$ (amino-ethyl-crotonic acid),

have the same ultimate composition as the four compounds which Schutzenberger obtained from albumin and to which he gave the name of leucéins—a knowledge of the constitution of which was, in his opinion, the only thing wanting to solve the problem as to the general structure of proteid matter. He consequently endeavoured in various ways, but unsuccessfully, to effect their synthesis.

Those which he was able to isolate presented the following characters[1] :—they crystallise with difficulty, or not at all, and are deliquescent ; they combine with baryta from which they are not completely disengaged by CO_2 ; they are not precipitated by mercuric nitrate. Though uncrystallisable themselves, they form crystalline compounds with the amido-derivatives of the fatty acids $C_nH_{2n+1}NO_2$.[2]

1. *Comptes Rendus*, t. LXXX, p. 238.
2. *Loc. cit.*, t. CI, p. 1267.

They differ, as he pointed out, from the leucines, amino-crotonic, amino-valerianic acid, etc., to which the general formula $C_nH_{2n+1}NO_2$ may be given, by containing two atoms less of hydrogen than the corresponding leucine, and may be represented by the general formula $C_pH_{2p-1}NO_2$. When combined we have—

$$\underset{\text{leucine}}{C_nH_{2n+1}NO_2} + \underset{\text{leucéine}}{C_pH_{2p-1}NO_2} = C_mH_{2m}N_2O_4 \text{ where } n + p = m.$$

These characters correspond with the salts of pyruvic acid and its homologues. The salts of pyruvic acid if prepared at the ordinary temperature can be obtained in a crystalline form; but if these aqueous solutions are heated to the boiling point, their character is changed and, on evaporation, gummy uncrystallisable salts remain behind. If an aqueous solution of the acid itself is evaporated by heat, a syrupy non-volatile acid is left behind, which when heated with HCl to 100° C. is resolved into CO_2 and pyrotartaric acid.[1]

$$\underset{\text{pyruvic acid}}{2\,CH_3\cdot CO\cdot COOH} = CO_2 + \underset{\text{pyrotartaric acid}}{CH_3\cdot CH(COOH)\cdot CH_2\cdot COOH}$$

Acetyl-acetic acid is a viscid fluid mixable with water, with a strong acid reaction. Its barium salt is amorphous and very soluble in water.

Methyl-acetyl-acetic acid or methyl-crotonic acid is a thick fluid mixable with water, which when heated is resolved into CO_2 and

$$CO\left\langle\begin{matrix}C_2H_5\\ CH_3\end{matrix}\right.$$ The barium salt is very soluble in water.[2]

Ethyl-crotonic acid possesses similar properties.

Having obtained from the 'résidu fixe' the various crystalline bodies to which I have referred, Schutzenberger then proceeds to resolve this latter into certain constituents. He arrives at the con-

1. Clermont, *Ber.*, Bd. 6, s. 72.

2. Beilstein, *Handb. d. Chemie*, 3te Aufl., B. IV, s. 601.

clusion, based on reasons which are fully set forth in his paper,[1] that the ' résidu fixe,' $C_{221}H_{435}N_{49}O_{105}$ is made up of the compounds :—

$C_9H_{11}NO_3$	... ...	tyrosine
$+\ 3\ C_9H_{16}N_2O_6$	... ...	mean of the strong amido-acids
$+\ 3\ C_9H_{20}N_2O_4$	... ...	leucines
$+\ 2\ C_7H_{18}N_2O_4$	... ...	gluco-proteins
$+16\ C_9H_{18}N_2O_4$	... ...	gluco-proteins
$C_{221}H_{435}N_{49}O_{105}$	... ...	*résidu fixe*

or in other words, when albumin is digested with five or six times its weight of barium hydrate for forty-eight hours, at a temperature of 180° C., it combines with 60 molecules of water and undergoes the following decomposition.[2]

$$\begin{aligned}&C_{210}H_{387}N_{65}O_{75}S_3 + 60\,H_2O \\ = \ &16\,NH_3 + 4\,(COOH)_2 + 3\,CO_2 + 4\,(CH_3 \cdot COOH) + S_3 \\ + \ &C_9H_{11}NO_3 + 3\,C_9H_{16}N_2O_6 + 3\,C_9H_{20}N_2O_4 \\ + \ &2\,C_7H_{14}N_2O_4 + 16\,C_9H_{18}N_2O_4\end{aligned}$$

I shall now endeavour to resolve these terms into their respective constituents. The last term in the equation $16\ C_9H_{18}N_2O_4$ is the one which presents the greatest difficulty in this respect for its formula may be satisfied by an indefinite number of combinations. The following considerations, however, furnish us with material assistance in arriving at a satisfactory solution.

In the first place we know that with regard to a large number of organic bodies, condensation of three molecules takes place forming compounds much more stable than those consisting of one molecule only : such compounds, for instance, as the cyanides, the cyanates, the cyanamides, the aldehydes, etc. It is consequently not an unwarrantable assumption that in such a body as albumin this ternary condensation or triple combination takes place. As a working hypothesis, at all events, it is useful, and we will assume that the constituents of $16\ C_9H_{18}N_2O_4$ exist in it either as single molecules or as compounds of three molecules or as multiples of three.

Secondly, we will assume that in this compound the leucines

1. See *Annales de Chimie*, 5me série, t. XVI, pp. 394-399.
2. *Loc. cit.*, pp. 385 and 398.

$C_nH_{2n+1}NO_2$ are combined with the corresponding leucéins $C_nH_{2n-1}NO_2$ forming the compounds $C_{2n}H_{4n}N_2O_4$.

Thirdly, Schutzenberger[1] points out that the amount of the compound $C_{12}H_{24}N_2O_4$ obtained from one molecule of albumin, and consisting of $C_6H_{13}NO_2 + C_6H_{11}NO_2$, is about 15 to 16 per cent.; $3\,(C_6H_{13}NO_2 + C_6H_{11}NO_2)$ corresponds to 14 per cent. If we subtract this from $16\,(C_9H_{18}N_2O_4)$, the remainder, he says, can only be resolved into 4 terms of C_5 and 22 of C_4, that is, into $2\,(C_5H_{11}NO_2 + C_5H_9NO_2)$ and $11\,(C_4H_9NO_2 + C_4H_7NO)$.[2] In other words—

$$\begin{aligned} 16\,(C_9H_{18}N_2O_4) &= 3\,(C_6H_{13}NO_2 + C_6H_{11}NO_2) \\ &+ 2\,(C_5H_{11}NO_2 + C_5H_9NO_2) \\ &+ 11\,(C_4H_9NO_2 + C_4H_7NO_2) \end{aligned}$$

In making this calculation, however, Schutzenberger leaves out of consideration the presence of alanine $C_3H_7NO_2$ or $CH_3 \cdot CH(NH_2) \cdot COOH$ which, as he shews,[3] is also contained in the 'résidu fixe'—'mais en petites quantités seulement.' One molecule of this combined with the corresponding leucéine $C_3H_5NO_2$ or $CH_3(NH)CH_2COOH$, forms the compound $C_3H_7NO_2 + C_3H_5NO_2 = C_6H_{12}N_2O_4$. Taking this into consideration, and that $2\,(C_4H_9NO_2 + C_4H_7NO_2)$ may represent $(C_5H_{11}NO_2 + C_5H_9NO_2) + (C_3H_7NO_2 + C_3H_5NO_2)$, the compound $16\,(C_9H_{18}N_2O_4)$ or $C_{144}H_{288}N_{32}O_{64}$ may be represented by

$$\begin{aligned} & 3\,(C_6H_{13}NO_2 + C_6H_{11}NO_2) &&= C_{36}H_{72}N_6O_{12} \\ + & 3\,(C_5H_{11}NO_2 + C_5H_9NO_2) &&= C_{30}H_{60}N_6O_{12} \\ + & 9\,(C_4H_9NO_2 + C_4H_7NO_2) &&= C_{72}H_{144}N_{18}O_{36} \\ + & C_3H_7NO_2 + C_3H_5NO_2 &&= C_6H_{12}N_2O_4 \\ \hline & && C_{144}H_{288}N_{32}O_{64} \end{aligned}$$

which satisfies the assumption made above as to the ternary combinations of the molecules. It also satisfies the requirement that amido-butyric acid and its complement $C_4H_7NO_2$ are the dominant factors of the 'résidu fixe,' which is in conformity with Schutzenberger's analysis.[4]

1. *Loc. cit.*, p. 397.
2. *Loc. cit.*, p. 398.
3. *Loc. cit.*, p. 358. It is found in the portion of the '*résidu fixe*' insoluble in alcohol.
4. *Loc. cit.*, p. 398.

The theory as to the ternary combination of the molecules can only be regarded as satisfactory if it holds good not only for this particular compound but for the general constitution of the albuminous molecule. The next term in the general equation 2 $C_7H_{14}N_2O_4$ would, therefore, at first sight seem to negative the theory, whereas the theory enables us to determine its composition.

The gluco-proteins 2 $C_7H_{14}N_2O_4$ may be regarded as the combination of two molecules of alanine, or amino-propionic acid, $CH_3 \cdot CH(NH_2) \cdot COOH$, with two molecules of the leucéin, imino-aceto-acetic acid $CH_3 \cdot (NH)CH_2 \cdot COOH$, which together form 2 $C_7H_{14}N_2O_4$ (gluco-proteins), and here the two molecules of amino-propionic acid with the one moluecle in the previous compound furnish the necessary triad.

If we regard 2 $(C_7H_{14}N_2O_4)$ as the combination of $\underbrace{2\,CH_3 \cdot CH_2 \cdot CH(NH_2)COOH}_{\text{amino-butyric acid}} + \underbrace{2\,CH_3 \cdot C(NH)COOH}_{\text{imino-pyruvic acid}}$ the same argument might be employed with respect to imido-pyruvic acid, but as will subsequently appear two molecules of amino-butyric acid would not be in accordance with it.

The next term 3 $C_9H_{20}N_2O_4$, or the leucines, is satisfied by the combination of

$$3C_4H_8(NH_2) \cdot COOH + 3C_3H_6(NH_2) \cdot COOH = 3C_9H_{20}N_2O_4$$

which, as it satisfies the ternary theory, is the one I adopt, though as

$$3C_5H_{11}NO_2 = C_6H_{13}NO_2 + C_5H_{11}NO_2 + C_4H_9NO_2 \text{ and}$$
$$3C_4H_9NO_2 = C_5H_{11}NO_2 + C_4H_9NO_2 + C_3H_7NO_2$$

many other combinations of these four amino acids would satisfy the formula.

The term $3C_9H_{16}N_2O_6$, representing what Schutzenberger terms 'les acides amidés forts,' contains the acids belonging to the glutamic series. Combining glutamic, aspartic and amino-malonic acids

respectively, with imino-ethyl-acetyl-acetic, imino-methyl-acetyl-acetic and imino-acetyl-acetic acids we have

$$\begin{array}{l} CH(NH_2)\cdot COOH \\ | \\ CH_2\cdot CH_2\cdot COOH \\ \text{glutamic acid} \end{array} + \underset{\text{imino-ethyl-acetyl-acetic acid}}{CH_3\cdot C(NH)\cdot CH(C_2H_5)\cdot COOH} = C_{10}H_{20}N_2O_6$$

$$\begin{array}{l} CH(NH_2)\cdot COOH \\ | \\ CH_2\cdot COOH \\ \text{aspartic acid} \end{array} + \underset{\text{imino-methyl-acetyl-acetic acid}}{CH_3 . C(NH)\cdot CH(CH_3)\cdot COOH} = C_9H_{16}N_2O_6$$

$$\begin{array}{l} CH(NH_2)\cdot COOH \\ | \\ COOH \\ \text{amino-malonic acid} \end{array} + \underset{\text{imino-acetyl-acetic acid}}{CH_3\cdot C(NH)\cdot CH_2\cdot COOH} = C_7H_{12}N_2O_6$$

$$\overline{C_{27}H_{48}N_6O_{18}}$$
$$\text{or } 3C_9H_{16}N_2O_6$$

Both glutamic and aspartic acids were obtained by Schutzenberger from the 'résidu fixe' and consequently must appear in the equation. Amino-malonic acid is not mentioned by him. The explanation I suggest is, that in the combination of amino-malonic acid and glutamic acid the compound would have the same molecular composition as aspartic acid, and as, according to Schutzenberger, the amido-acids of the same series show a great tendency to crystallise together (see p. 230), we can have

$$\underset{\text{glutamic acid}}{C_5H_9NO_4} + \underset{\text{amino-malonic acid}}{C_3H_5NO_4} = 2\,(C_4H_7NO_4)$$

and in this way its presence might be overlooked.

Taking, then, the formula $C_{240}H_{397}N_{65}O_{75}S_3$ for the composition of albumin (differing from Schutzenberger's by H_{10}) we find that by the action of BaH_2O_2 at 180° C. it combines with 60 molecules of H_2O and is resolved first into—

(i) $16\,NH_3 + 4\,(COOH)_2 + 3\,CO_2 + 4\,(CH_3 . COOH) + S_3 + H_{10}$
(ii) $+ C_9H_{11}NO_3$
(iii) $+ 3\,C_9H_{16}N_2O_6$
(iv) $+ 3\,C_9H_{18}N_2O_4$
(v) $+ 2\,C_7H_{14}N_2O_4$
(vi) $+ 16\,C_9H_{18}N_2O_4$

and resolving (iii), (iv), (v) and (vi) into their components, the whole may be represented in the following tabular form :—

THE COMPLETE HYDROLYTIC DECOMPOSITION OF EGG ALBUMIN WITH BARIUM HYDRATE AT 180° C.

(i)	(ii)	(iii)	(iv)	(v)	(vi)
16 NH_3 +4 $(COOH)_2$ oxalic acid +3 CO_2 +4 $CH_3 . COOH$ acetic acid +S+2 SH_2 + H_6	$C_6H_4(OH) . CH_2 . CH(NH_2)$ COOH tyrosine	$CH(NH_2) . COOH$ | $CH_2 . CH_2 . COOH$ glutamic acid $CH(NH_2) . COOH$ | $CH_2 . COOH$ aspartic acid $CH(NH_2) . COOH$ | COOH amino-malonic acid	3 $CH_3 . (CH_2)_2 . CH(NH)_2 . COOH$ *a* amino-valerianic acid 3 $CH_3 . CH_2 . CH(NH_2) . COOH$ *a* amino-butyric acid	2 $CH_3 . CH(NH_2) . COOH$ *a* amino-propionic acid	3 $CH_3(CH_2)_3 . CH(NH_2) . COOH$ *a* amino-caproic acid 3 $CH_3 . (CH_2)_2 . CH(NH_2) . COOH$ *a* amino-valerianic acid 9 $CH_3 . CH_2 . CH(NH_2) . COOH$ *a* amino-butyric acid $CH_3 . CH(NH_2) . COOH$ *a* amino-propionic acid
		$CH_3 . C(NH) . CH(C_2H_5) . COOH$ imino-ethyl-acetyl-acetic acid $CH_3 . C(NH) . CH(CH_3) . COOH$ imino-methyl-acetyl-acetic acid $CH_3 . C(NH) . CH_2 . COOH$ imino-acetyl-acetic acid		2 $CH_3 . C(NH) . CH_2 . COOH$ imino-acetyl-acetic acid	3 $CH_3 . C(NH) . CH(C_2H_5) . COOH$ imino-ethyl-acetyl-acetic acid 3 $CH_3 . C(NH) . CH(CH_3) . COOH$ imino-methyl-acetyl-acetic acid 9 $CH_3 . C(NH) . CH_2 . COOH$ imino-acetyl-acetic acid $CH_3 . C(NH) . COOH$ imino-pyruvic acid
$C_{19}H_{82}N_{16}O_{30}S_3$ Schutenzberger's polynomial with H_{10} added	$C_9H_{11}NO_3$ tyrosine	$C_{27}H_{48}N_6O_{18}$ or 3 $C_9H_{16}N_2O_6$ acids amidés forts	$C_{27}H_{60}N_6O_{12}$ or 3 $C_9H_{20}N_2O_4$ leucines	$C_{14}H_{28}N_4O_8$ or 2 $C_7H_{14}N_2O_4$ gluco-proteins	$C_{144}H_{288}N_{32}O_{64}$ or 16 $C_9H_{18}N_2O_4$ gluco-proteins

$C_{221}H_{435}N_{49}O_{105}$
résidu fixe

$C_{240}H_{517}N_{65}O_{135}S_3$
or $C_{240}H_{397}N_{65}O_{75}S_3 + 60\ H_2O$
albumin

ON THE SYNTHESIS OF LIVING ALBUMIN

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Having under the conditions given in the preceding papers determined the products which result from the complete hydrolysis of albumin, I will now proceed to consider what is the constitution of albumin itself, or in other words, what are the respective constituents of the albuminous molecule from which these products are derived.

In dealing with the origin of lactic acid I have already indicated the way in which, in the earlier stages of the genesis of organic matter in plants, the following substances may be formed, hydrocyanic acid; its condensed product amino-malonic nitrile, with its derivative tartronic nitrile; acetic anhydride; pyruvic nitrile; and methyl-tartronic nitrile. The series of changes by which we can proceed to the formation of the various fatty aldehydes, commencing with the lowest, namely, formic aldehyde, have also been referred to.[1] It remains now to consider what are the antecedents of tyrosine and in what form they exist in protein. Secondly, in what form do the antecedents of the amino-fatty acids exist therein, and lastly what are the antecedents of aspartic and glutamic acids.

The answer to these questions will be obtained by considering the synthesis of the respective bodies.

On the Antecedents of Tyrosine in Living Protein

On page 199 I have already indicated the method by which the synthesis of tyrosine may be effected from *p.* oxybenzaldehyde, amino-malonic nitrile, and 2 HCN. Practically the result is—

$$\underset{p.\text{ oxybenzaldehyde}}{OH \cdot C_6H_4 \cdot CHO} + \underset{\text{amino-malonic nitrile}}{CH(NH_2) \cdot (CN)_2} + 2\,HCN + 7H_2O$$

$$= \underset{\text{tyrosine}}{p.\,OH \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2)COOH} + CO_2 + 2\,NH_3 + \underset{\text{ammonium oxalate}}{(COO)_2\,(NH_4)_2}$$

1. See pages 195-8.

By similar reactions we may from *p.* amino-benzaldehyde obtain *p.* amino-phenyl alanine *p.* $NH_2 \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ the hydrochloride of which treated with a single molecular equivalent of $NaNO_2$ at 0° C. and then boiled yields tyrosine; but if an excess of $NaNO_2$ is used the compound is decomposed into tyrosine and oxyphenyl lactic acid,[1] $OH \cdot C_6H_4 \cdot CH_2 \cdot CH(OH) \cdot COOH$. Now when tyrosine is taken into the animal system it is converted into hydro-para-coumaric acid and *p.* oxy-phenyl-acetic acid[2]. With this exception, however, all other para derivatives of benzene when taken into the animal system are immediately excreted, with very little change, in the urine[3], so that it is improbable that either *p.* oxy-benzoic aldehyde or *p.* amino-benzaldehyde exist in living protein. On the other hand *o.* amino-benzaldehyde under certain conditions combines with aldehydes and ketones to form chinoline derivatives[4]

$$C_6H_4\begin{cases}CHO\\NH_2\end{cases} + \begin{matrix}CH_2-R\\ |\\ CO-R^1\end{matrix} = C_6H_4\begin{cases}CH=C\cdot R\\ \quad\;\;\; |\\ N=\;\; C\cdot R^1\end{cases} + H_2O$$

and this suggests that *o.* amino benzaldehyde may be a constituent of living protein, since chinoline and its derivatives are obtained by the distillation of certain vegetable alkaloids which are derivatives of vegetable albumin. Some of the ortho-derivatives of benzene, moreover, under certain conditions, can be transformed into the para-compounds; *o.* oxybenzoic acid for instance when heated with potash to 220° is converted in some degree into *p.* oxybenzoic acid.[5]

I shall therefore assume that in the living protein amino-benzaldehyde exists in the *ortho*-form, and that in some way it is converted into the *para*-form before entering into the formation of tyrosine. In living protein then we have as the antecedents of tyrosine, amino-oxy-benzaldehyde, amino-malonic nitrile and hydrocyanic acid—

$$NH_2 \cdot C_6H_4 \cdot CHO + CH(NH_2) \cdot (CN)_2 + 2\,HCN$$

which with 8 H_2O form

$$\underset{\text{tyrosine}}{p.\ OH \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2)COOH} + CO_2 + 5\,NH_3 + \underset{\text{oxalic acid}}{(COOH)_2}$$

1. Friedländer, *Annal.*, Bd. CCXIX, s. 223, u. Bd. CCXXIX, s. 227.
2. Blendermann, *Zeitsch. f. phys. Chemie*, Bd. VI, s. 234.
3. Schrotten, *ibid.*, Bd. VII, s. 23.
4. Friedländer, *Berichte*, Bd. XV, s. 2574, Bd. XVI, s. 1833, Bd. XXV, s. 1752.
5. Kolbe, *Journ. f. praktische Chemie* (2), Bd. XI, s. 24.

On the Synthesis of the Amino-Fatty Acids

Amino - Caproic Acid $(CH_3 \cdot (CH_2)_3 \cdot CH(NH_2)COOH)$.— If valerianic aldehyde is mixed with aqueous ammonia the aldehyde is converted into valeral ammonia and this digested with hydrocyanic acid and hydrochloric acid, is converted into leucine.

$$\underset{\text{valerianic aldehyde}}{CH_3(CH_2)_3 \cdot CHO} + NH_3 = \underset{\text{valeral ammonia}}{C_6H_4 \cdot CH(NH_2) \cdot OH}$$

and

$$C_6H_4 \cdot CH(NH_2) \cdot OH + HCN = \underset{\substack{\text{normal amino-caprioic acid} \\ \text{or leucine}}}{CH_3 \cdot (CH_2)_3 \cdot CH(NH_2)COOH} + NH_3$$

This is the usual way of obtaining leucine synthetically. Tiemann, however, has shown[1] that the amido-acids, both of the fatty and aromatic series, may be obtained by converting the aldehydes and ketones into cyan-alcohols, then into amino-nitriles or cyan-amides and thence into the amido acids. We may consequently have the following changes :—

$$\underset{\text{valerianic aldehyde}}{CH_3 \cdot (CH_2)_3CHO} + HCN = \underset{\text{valerianic nitrile}}{CH_3 \cdot (CH_2)_3CH\begin{cases} OH \\ CN \end{cases}}$$

1. *Ber.* XIV, s. 1985. 'The amido-acids of the fatty series are easily obtained by the familiar reactions which take place on treating aldehyde ammonia with hydrochloric and hydrocyanic acids, and which led Strecker to the discovery of alanine. . . The reactions indicated by Strecker take place unquestionably according to the following general formulae :—

$$R \cdot CH\begin{cases} NH_2 \\ OH \end{cases} + HCN = R \cdot CH\begin{cases} NH_2 \\ CN \end{cases} + H_2O$$

$$\text{and } CH\begin{cases} NH_2 \\ CN \end{cases} + 2\,H_2O + HCl = R \cdot CH(NH_2)COOH + NH_4Cl.$$

The question arises, whether the cyanamide $R \cdot CH\begin{cases} NH_2 \\ CN \end{cases}$ could not be obtained more readily from the cyanhydrides of the aldehydes $R \cdot CH\begin{cases} OH \\ CN \end{cases}$ by digesting them with ammonia, expecting the ultimate change to be as follows :—

$$R \cdot CH\begin{cases} OH \\ CN \end{cases} + NH_3 = R \cdot CH\begin{cases} NH_2 \\ CN \end{cases} + H_2O$$

The truth of this supposition has been confirmed by experiment.'—*Ber.* XIII, s. 382.

$$C_4H_9 \cdot CH \left\{\begin{matrix} OH \\ CN \end{matrix}\right. + NH_3 = \underset{\text{amino-caproic nitrile}}{C_4H_9 \cdot CH \left\{\begin{matrix} NH_2 \\ CN \end{matrix}\right.} + H_2O$$

$$C_4H_9 \cdot CH \left\{\begin{matrix} NH_2 \\ CN \end{matrix}\right. + 2\,H_2O = \underset{\substack{\text{amino-caproic acid} \\ \text{or leucine}}}{C_4H_9 \cdot CH \left\{\begin{matrix} NH_2 \\ COOH \end{matrix}\right.} + NH_3$$

which by dehydration is converted into the anhydride $C_4H_9 \cdot CH \left\langle \begin{matrix} NH \\ | \\ CO \end{matrix} \right.$

This method is applicable generally to the production of the amino-acids and we have

$$R \cdot CHO \rightarrow R \cdot CH \left\langle \begin{matrix} OH \\ CN \end{matrix} \right. \rightarrow R \cdot CH \left\langle \begin{matrix} NH_2 \\ CN \end{matrix} \right. \rightarrow R \cdot CH \left\langle \begin{matrix} NH_2 \\ COOH \end{matrix} \right. \rightarrow R \cdot CH \left\langle \begin{matrix} NH \\ | \\ CO \end{matrix} \right.$$

Practically, however, we may regard the cyan-alcohols as the constituents in living albumin, which are the antecedents of, and by hydrolysis are converted into the amino-fatty acids, since omitting the intermediate formation of the nitrile of the amino acid we have

$$\underset{\text{cyanalcohol}}{R \cdot CH \left\langle \begin{matrix} OH \\ CN \end{matrix} \right.} + H_2O = \underset{\text{amino acid}}{R \cdot CH \left\langle \begin{matrix} NH_2 \\ COOH \end{matrix} \right.}$$

Tiemann has also shewn[1] that by treating the di-ethyl, ethyl-methyl, and di-methyl ketones in the same manner, other iso-amino acids are formed—

$$(C_2H_5)_2 \cdot CO + HCN = (C_2H_5)_2 \cdot C \left\{\begin{matrix} OH \\ CN \end{matrix}\right. \rightarrow (C_2H_5)_2 \cdot C \left\{\begin{matrix} NH_2 \\ COOH \end{matrix}\right.$$

$$(CH_3)_2 \cdot CO \cdot + HCN \; (CH_3)_2 \cdot C \left\{\begin{matrix} OH \\ CN \end{matrix}\right. \rightarrow (CH_3)_2 \cdot C \left\{\begin{matrix} NH \\ COOH \end{matrix}\right.$$

These amino-fatty acids can also be prepared[2] in the same way as tyrosine by combining the fatty aldehydes by means of Perkin's

1. *Berichte*, Bd. XIV, s. 1975.
2. E. Erlenmeyer, Jnr., *Annal. d. Chemie*, Bd. CCCVII, s. 74.

reaction with those derivatives of glycocoll which have one of the hydrogen atoms of the NH_2 replaced by an acid radicle such as

$$\text{acetyl-glycocoll } CH_2\left\langle\begin{array}{l} NH\cdot CO\cdot CH_3, \\ COOH \end{array}\right.$$

$$\text{or hippuric acid } CH_2\left\langle\begin{array}{l} NH\cdot CO\cdot C_6H_5 \\ COOH \end{array}\right.$$

$$R\cdot CHO + CH_2\left\langle\begin{array}{l} NH\cdot CO\cdot C_6H_5 \\ COOH \end{array}\right. = R\cdot CH:C\left\langle\begin{array}{l} NH\cdot CO\cdot C_6H_5 \\ COOH \end{array}\right. + H_2O$$

$$= R\cdot CH:\underbrace{C—N}_{CO}—CO\cdot C_6H_5 + 2\,H_2O$$

forming a lactimide which heated with dilute aqueous solution of soda is transformed into an acid. This on reduction becomes $R\cdot CH_2\cdot CH\left\langle\begin{array}{l} NH\cdot CO\cdot C_6H_5 \\ COOH \end{array}\right.$ and can then be resolved into the α-amino acid $R\cdot CH\cdot CH_2\left\langle\begin{array}{l} NH_2 \\ COOH, \end{array}\right.$ and benzoic acid $C_6H_5\cdot COOH$.

On the Synthesis of Aspartic and Glutamic Acids.—

$$\begin{array}{l} CH(NH_2)\cdot COOH \\ | \\ CH_2\cdot COOH \end{array} \quad\text{and}\quad \begin{array}{l} CH(NH_2)\cdot COOH \\ | \\ CH_2\cdot CH_2\cdot COOH \end{array}$$

The lowest member of this series is amino-malonic acid $\begin{array}{l} CH(NH_2)\cdot COOH \\ | \\ COOH \end{array}$ the nitrile of which $\begin{array}{l} CH(NH_2)\cdot CN \\ | \\ CN \end{array}$ is formed as shewn on page 197, from the condensation of three molecules of HCN, and by desamination should be converted into tartronic nitrile. Theoretically it should be possible to convert these two bodies respectively into amino-malonic acid and tartronic acid. Hitherto this has not been accomplished.

We have here, however, a demonstration of the existence of the nitrile of the lowest member of the aspartic series and this is a strong argument in favour of the existence of the nitriles of the two other members. The facts also that the antecedents of the amino-fatty acids are the fatty aldehydes combined with HCN, and that the antecedent of tyrosine is an aromatic aldehyde render it most probable that aspartic and glutamic acids have a similar origin. I shall now endeavour to shew how, in this way, their synthesis may possibly be accomplished.

Synthesis of Aspartic Acid.—Cyan-acet-aldehyde $CH_2(CN) \cdot CHO$ can be obtained from chloracetal $CH_2Cl \cdot CH(C_2H_5O)_2$ or, chlor-acet-aldehyde $CH_2Cl \cdot CHO$ by converting the latter into iodo-aldehyde, and thence into the cyanogen compound. Treating this compound with HCN and then with NH_3 we should obtain the corresponding cyanamide—

$$\underset{\text{cyanacetaldehyde}}{CH_2(CN) \cdot CHO} + HCN = \underset{\text{oxy-succinic nitrile}}{CN \cdot CH_2 \cdot CH \left\langle \begin{matrix} OH \\ CN \end{matrix} \right.}$$

$$CN \cdot CH_2 \cdot CH \left\langle \begin{matrix} OH \\ CN \end{matrix} \right. + NH_3 = \underset{\text{aspartic nitrile}}{CN \cdot CH_2 \cdot CH(NH_2)CN} + H_2O$$

which acted upon by acids or alkalies would be converted into asparagine or aspartic acid—

$$CN \cdot CH_2 \cdot CH(NH_2)CN + 3\,H_2O = \underset{\text{asparagine}}{\begin{matrix} CH(NH_2) \cdot CO \cdot NH_2 \\ | \\ CH_2 \cdot COOH \end{matrix}} + NH_3$$

$$\text{or with} \quad + 4\,H_2O = \underset{\text{aspartic acid}}{\begin{matrix} CH(NH_2)COOH \\ | \\ CH_2 \cdot COOH \end{matrix}} + NH_3$$

consequently if oxysuccinic nitrile, which, I suggest, is the antecedent of aspartic acid in living protein, is heated in a sealed tube with BaH_2O_2 the following reaction would ensue :—

$$\underset{\text{oxysuccinic nitrile}}{CN \cdot CH_2 \cdot CH(OH)CN} + 3H_2O = \underset{\text{aspartic acid}}{COOH \cdot CH_2 \cdot CH(NH_2)\,COOH} + NH_3$$

If, however, the cyan-acet-aldehyde is first combined with NH_3 we should have—

$$CN \cdot CH_2 \cdot CHO + NH_3 = CN \cdot CH_2 \cdot CH \begin{cases} NH_2 \\ OH \end{cases}$$

which reduced by nascent hydrogen (H_4) becomes

$$= CH_2(NH_2) \cdot CH_2 \cdot CH \begin{cases} NH_2 \\ OH \end{cases}$$

Combining this with HCN we obtain $CH_2(NH_2) \cdot CH_2 \cdot CH(NH_2)CN$ which on saponification becomes $\underset{\text{diamino-butyric acid}}{CH_2(NH_2) \cdot CH_2 \cdot CH(NH_2)COOH}$

Moreover, if by the Perkin's reaction we combine cyan-acetic-aldehyde with acetyl or benzoyl glycocoll (derived from amino-malonic nitrile) as in the synthesis of tyrosine, the following reactions should ensue :—

$$\begin{aligned} &CN \cdot CH_2 \cdot CHO + CH_2(NH \cdot CO \cdot C_6H_5)COOH \\ &= CN \cdot CH_2CH : C(NH \cdot CO \cdot C_6H_5)COOH \end{aligned}$$

which on reduction with H_6 becomes

$$\underset{\text{benzoyl ornithin}}{CH_2(NH_2) \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot CO \cdot C_6H_5)COOH^*}$$

and is then resolved into

$$\underset{\text{1-4 di-amino valerianic acid or ornithin}}{CH_2(NH_2) \cdot CH_2 \cdot CH_2 \cdot CH(NH_2)COOH} + \underset{\text{benzoic acid}}{C_6H_5 \cdot COOH}$$

By bacterial action Ellinger[1] succeeded in converting this into

$$\underset{\text{putrescine}}{CH_2(NH_2)CH_2 \cdot CH_2 \cdot CH_2(NH_2)}$$

By combining cyanamide with ornithin, argenin is formed.

The Synthesis of Glutamic Acid.—We may assume that with β cyanpropionic aldehyde $CH_2(CN) \cdot CH_2 \cdot CHO$ similar reactions will take place to those with cyan-acet-aldehyde. Treating it with

* Which is excreted by birds after the ingestion of benzoic acid (Kossel).

1. *Zeitsch. f. phys. Chemie*, Bd. XXIX, s. 334.

HCN, and then with NH_3, the cyan-alcohol would first be formed and then the cyan-amide or

$$CN \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CN$$
glutamic nitrile

which by saponification would be converted into

$$COOH \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH + 2\ NH_3$$
glutamic acid .

If, however, the cyan-propionic aldehyde is first combined with NH_3 and then undergoes reduction the following reactions will take place :—

$$CN \cdot CH_2 \cdot CH_2 \cdot CHO + NH_3 = CN \cdot CH_2 \cdot CH_2 \cdot CH\Big\langle{}^{OH}_{NH_2}$$

which reduced by

$$H_4 = CH_2(NH_2) \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH\Big\langle{}^{OH}_{NH_2}$$

combining this with HCN, and then saponifying, we again obtain ornithin or

$$CH_2(NH_2) \cdot CH_2 \cdot CH_2 \cdot CH(NH_2)COOH$$
1-4 diamino-valerianic acid

On the other hand if we combine the cyan-propionical dehyde with acetyl or benzoyl-glycocoll by the Perkin's reaction and then reduce the resulting compound we should obtain

$$CH_2(NH_2) \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH$$
1-5 diamino-normal caproic acid
or lysin

which combined with cyanamide forms Drechsel's lysatinine.

By bacterial action also lysin is converted into cadaverine

$$NH_2(CH_2)_5 \cdot NH_2$$

It follows from this that aspartic acid and ornithin have a common origin, which is also the case with glutamic acid and lysine. Furthermore, that oxy-succinic or malic nitrile and oxy-glutaric nitrile are the antecedents in living albumin of aspartic acid and glutamic acid respectively, since by heating them in a sealed tube with BaH_2O_2 these two acids would be produced.

Finally, we have to determine the antecedents in the protein molecule, of Schutzenberger's polynomial;

$$16NH_3 \cdot + \underset{\text{acetic acid}}{4\,CH_3 \cdot COOH} + 3\,CO_2 + \underset{\text{oxalic acid}}{4\,(COOH)_2} + S_3 + [H_{10}]$$

The very simplicity of this polynomial makes it difficult to determine its antecedents, for the possible ways in which they may arise may truly be said to be innumerable. It is, however, unnecessary for me to discuss the possibilities. I shall content myself with stating the result at which I have arrived, briefly indicating the grounds which have led me to it.

In describing the synthesis of tyrosine I have shewn that its antecedents are—

$$\underset{\substack{\text{amino-malonic}\\ \text{nitrile}}}{CH(NH_2)(CN)_2} + \underset{\substack{\text{amido-benzoic}\\ \text{aldehyde}}}{NH_2 \cdot C_6H_4 \cdot CHO} \cdot + 2(HCN)$$

which with eight molecules of H_2O give

$$\underset{\text{tyrosine}}{OH \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2)COOH} + \underset{\substack{\text{oxalic}\\ \text{acid}}}{(COOH)_2} + CO_2 + 5\,NH_3$$

thus furnishing $(COOH)_2 + CO_2 + 5\,NH_3$ towards the polynomial. Again in describing the synthesis of glutamic, aspartic and amino-malonic acids I have represented these as resulting from oxyglutaric, malic and tartronic nitriles. These under the action of baryta in a sealed tube become respectively—

$$\begin{array}{l} CH\!\left\langle\begin{array}{l}OH\\ CN\end{array}\right. \\ | \\ CH_2 \cdot CH_2 \cdot CN \end{array} + 3\,H_2O = \underset{\text{glutamic acid}}{\begin{array}{l} CH\!\left\langle\begin{array}{l}NH_2\\ COOH\end{array}\right. \\ | \\ CH_2 \cdot CH_2 \cdot COOH \end{array}} + NH_3$$

$$\begin{array}{l} CH\!\left\langle\begin{array}{l}OH\\ CN\end{array}\right. \\ | \\ CH_2 \cdot CN \end{array} + 3H_2O = \underset{\text{aspartic acid}}{\begin{array}{l} CH\!\left\langle\begin{array}{l}NH_2\\ COOH\end{array}\right. \\ | \\ CH_2 \cdot COOH \end{array}} + NH_3$$

$$\begin{array}{l} CH\!\left\langle\begin{array}{l}OH\\ CN\end{array}\right. \\ | \\ CN \end{array} + 3\,H_2O = \underset{\text{amino-malonic acid}}{\begin{array}{l} CH\!\left\langle\begin{array}{l}NH_2\\ COOH\end{array}\right. \\ | \\ COOH \end{array}} + NH_3$$

furnishing three more molecules of NH_3. The remaining portion of the polynomial—

$$8\,NH_3 + 4\,CH_2 \cdot COOH + 2\,CO_2 + 6\,H \cdot COOH + 2\,SH_2 + S$$

is, therefore, all that remains to be considered.

It has been shewn that all the compounds hitherto dealt with may be derived from the cyanides or nitriles. It is not improbable, therefore, that the sulphur in albumin is present also as a cyanide, namely, as sulpho-cyanide, and this assumption is rendered more probable as this compound is found, though in small quantities, in the saliva, and is also excreted in the urine.[1]

Sulphocyanic acid $CN \cdot SH$ is very unstable, and is quickly resolved into hydrocyanic acid and persulphocyanic acid[2] which under

$$3\,CN \cdot SH = HCN + C_2N_2H_2S_3$$

certain conditions are resolved as follows :—[3]

$$HCN + 2\,H_2O = NH_3 + H \cdot COOH$$

and

$$C_2H_2H_2S_3 + 4\,H_2O = 2\,CO_2 + 2\,NH_3 + 2\,SH_2 + S$$

This is the form, then, in which I suggest the sulphur exists in the albuminous molecule, and which furnishes these constituents of the polynomial.

We have now only to determine the origin of the remaining terms

$$5\,NH_3 + 4\,CH_3 \cdot COOH + 5\,H \cdot COOH$$

In a previous paper (see page 200) I endeavoured to show that diacetyl-dicyanide was a constituent of protein, being the antecedent of methyl-tartronic nitrile. Now diacetyl-dicyanide acted upon by alkalies in the cold is resolved into NH_3, HCN and $CH_3 \cdot COOH$, and when the temperature is raised we have

$$(CH_3 \cdot CO \cdot CN)_2 + 6\,H_2O = 2\,CH_3 \cdot COOH + 2\,NH_3 + 2\,H \cdot COOH$$

I have also shown that 3 HCN and $(CH_3 \cdot CO)_2O$ are among the

1. Gschleiden, *Jahresb. uber die Fortsch. d. Chemie*, s. 1001, 1877.
2. *Watt's Dictionary of Chemistry*, Vol. V, p. 505.
3. *Ibid.*, Vol. IV, p. 379.

earliest products in the genesis of protein. These two substances acted upon by BaH_2O_2 are hydrolysed as follows :—

$$\begin{aligned} 3\,HCN + 6\,H_2O &= 3\,H\cdot COOH + 3\,NH_3\cdot \\ (CH_3\cdot CO)_2O + H_2O &= 2\,CH_3\cdot COOH \end{aligned}$$

The following compounds, therefore, may be regarded as the antecedents of the polynomial :—

$$\begin{array}{llllll} 3\,HCN & + 6\,H_2O = & 3\,NH_3 + 3\,H\cdot COOH & & & \\ 3\,HCNS & + 6\,H_2O = & 3\,NH_3 + \,H\cdot COOH & & & + 2\,CO_2 + 2\,SH_2 + S \\ (CH_3\cdot CO)_2O & + \,H_2O = & & & 2\,CH_3\cdot COOH & \\ (CH_3\cdot CO\cdot CN)_2 & + 6\,H_2O = & 2\,NH_3 + 2\,H\cdot COOH & + & 2\,CH_3\cdot COOH & \end{array}$$

that is to say—

$$\begin{aligned} &3\,HCN + 3\,CNHS + (CH_3\cdot CO)_2O + (CH_3\cdot CO\cdot CN)_2 + 19\,H_2O \\ &= 8\,NH_3 + 6\,H\cdot COOH + 4\,CH_3\cdot COOH + 2\,CO_2 + 2\,SH_2 + S \end{aligned}$$

which with the terms $CO_2 + 5\,NH_3 + (COOH)_2$ furnished by the hydrolysis of the antecedents of tyrosine, and with $3\,NH_3$ resulting frcm the hydrolytic decomposition of tartronic, malic and oxyglutaric nitriles in a sealed tube make up—

$$16\,NH + 4\,CH_3\cdot COOH + 3\,CO_2 + (COOH)_2 + 6\,H\cdot COOH + 2\,SH_2 + S$$

which, as $2\,H\cdot COOH$ heated in a sealed tube with BaH_2O_2 is converted into $H_2 + (COOH)_2$ becomes

$$16\,NH_3 + \underset{\text{acetic acid}}{4\,CH_3\cdot COOH} + 3\,CO_2 + \underset{\substack{\text{oxalic}\\ \text{acid}}}{4\,(COOH)_2} + H_6 + 2\,SH_2 + S$$

From these data the composition of living albumin may be represented in the following tabular form :—

COMPOSITION OF LIVING ALBUMIN

(i)	(ii)	(iii)	(iv)	(v)	(vi)
$3\,HCN$	$(NH_2)C_6H_4\cdot CHO$ amino-benzaldehyde	$\begin{matrix}CH(OH)\cdot CN\\ \vert \\ CH_2\cdot CH_2\cdot CN\end{matrix}$ oxy-glutaric nitrile			$3\,CH_3\cdot(CH_2)_3\cdot CH\left\langle\begin{matrix}OH\\CN\end{matrix}\right.$ pentene cyan-alcohol
$3\,CNHS$ thio-cyanic acid	$CH(NH_2)(CN)_2$ amino malonic nitrile $+\,2\,HCN$		$3\,CH_3\cdot(CH_2)_2\cdot CH\left\langle\begin{matrix}OH\\CN\end{matrix}\right.$ butene cyan-alcohol		$3\,CH_3\cdot(CH_2)_2\cdot CH\left\langle\begin{matrix}OH\\CN\end{matrix}\right.$ butene cyan-alcohol
$(CH_3\cdot CO)_2O$ acetic anhydride		$\begin{matrix}CH(OH)\cdot CN\\ \vert \\ CH_2\cdot CN\end{matrix}$ malic or oxy-succinic nitrile	$3\,CH_3\cdot CH_2\cdot CH\left\langle\begin{matrix}OH\\CN\end{matrix}\right.$ propylidene cyan-alcohol		$9\,CH_3\cdot CH_2\cdot CH\left\langle\begin{matrix}OH\\CN\end{matrix}\right.$ propylidene cyan-alcohol
$(CH_3\cdot CO\cdot CN)_2$ diacetyl-dicyanide		$\begin{matrix}CH(OH)\cdot CN\\ \vert \\ CN\end{matrix}$ tartronic, or oxy-malonic nitrile		$2\,CH_3\cdot CH\left\langle\begin{matrix}OH\\CN\end{matrix}\right.$ ethylidene cyan-alcohol	$CH_3\cdot CH\left\langle\begin{matrix}OH\\CN\end{matrix}\right.$ ethylidene cyan-alcohol
		$CH_3\cdot C(NH)\cdot CH(C_2H_5)\cdot COOH$ imino-ethyl-acetyl-acetic acid			$3\,CH_3\cdot(NH)\cdot CH(C_2H_5)\cdot COOH$ imino-ethyl-acetyl-acetic acid
		$CH_3\cdot C(NH)\cdot CH(CH_3)\cdot COOH$ imino-methyl-acetyl-acetic acid			$3\,CH_3\cdot C(NH)\cdot CH(CH_3)\cdot COOH$ imino-methyl-acetyl-acetic acid
		$CH_3\cdot C(NH)\cdot CH_2\cdot COOH$ imino-acetyl-acetic acid		$2\,CH_3\cdot C(NH)\cdot CH_2\cdot COOH$ imino-aectyl-acetic acid	$9\,CH_3\cdot C(NH)\cdot CH_2\cdot COOH$ imino-acetyl-acetic acid
					$CH_2\cdot C(NH)\cdot COOH$ imino-pyruvic acid
$C_{16}H_{18}N_8O_5S_3 + 19\,H_2O = C_{16}H_{56}N_8O_{24}S_3$	$C_{12}H_{12}N_6O + 8\,H_2O = C_{12}H_{28}N_6O_9$	$C_{27}H_{39}N_9O_9 + 9\,H_2O = C_{27}H_{57}N_9O_{18}$	$C_{27}H_{48}N_6O_6 + 6\,H_2O = C_{27}H_{60}N_6O_{12}$	$C_{14}H_{24}N_4O_6 + 2\,H_2O = C_{14}H_{28}N_4O_8$	$C_{144}H_{256}N_{32}O_{48} = C_{240}H_{397}N_{65}O_{75}S_3$; $+\,16\,H_2O = +\,60\,H_2O$; $C_{144}H_{288}N_{32}O_{64} = C_{240}H_{517}N_{65}O_{75}S_3$

On the Composition of Dead Albumin

In my Croonian Lectures in 1886 delivered at the Royal College of Physicians,[1] and again in 1897[2] I suggested that in dead proteid, the antecedents of the amino-fatty acids are their anhydrides, a triple union of each taking place :—

$$3\,CH_2\left\langle\begin{array}{l} NH \\ | \\ CO \end{array}\right. = \left.\begin{array}{l} CH_2—NH—CO—CH_2 \\ | \\ CO—NH—CH_2—CO \end{array}\right\rangle NH$$

glycin anhydride

or generally—

$$3\,R\cdot CH\left\langle\begin{array}{l} NH \\ | \\ CO \end{array}\right. = \left.\begin{array}{l} R\cdot CH—NH—CO—R\cdot CH \\ | \\ CO—NH—R\cdot CH—CO \end{array}\right\rangle NH$$

compounds which, when the ring is broken, are now known as polypeptides.[3]

Further in accordance with Pflüger's[4] view that ammonium cyanate is the type of living and urea of dead nitrogen, and that the conversion of the former into the latter is an image of the essential change which takes place when a living proteid dies, I suggested that when $R\cdot CH\left\langle\begin{array}{l} NH \\ | \\ CO \end{array}\right.$ in the above triple molecule becomes part of living tissue it is transformed into the cyan-alcohol $R\cdot CH\left\langle\begin{array}{l} OH \\ CN \end{array}\right.$ and *vice versa*.

On this assumption the cyan-alcohols in columns (vi), (v), and (iv) of the table giving the composition of living albumin, are in dead albumin transformed into their respective anhydrides.

$$3\,R\cdot CH\left\langle\begin{array}{l} OH \\ CN \end{array}\right. \rightarrow 3\,R\cdot CH\left\langle\begin{array}{l} OH \\ CONH_2 \end{array}\right. \rightarrow 3R\cdot CH\left\langle\begin{array}{l} NH_2 \\ COOH \end{array}\right. \rightarrow 3\,R\cdot CH\left\langle\begin{array}{l} NH \\ | \\ CO \end{array}\right.$$

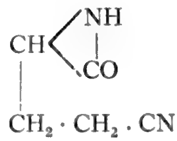
1. 'On some points in the Pathology of Rheumatism, Gout and Diabetes.' Deighton, Bell & Co., Cambridge, 1887.

2. 'On the Synthesis of Dead and Living Proteid.' Deighton, Bell & Co., Cambridge, 1897.

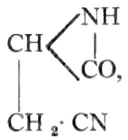
3. For the mode of preparation of these polypeptides see E. Fischer and others, *Ber.*, 1901, Bd. XXXIV, s. 2868; Bd. XXXV, 1095; Bd. XXXVI, sn. 2094, 2106, 2592, etc.

4. *Pflüger's Archiv.*, Bd. X, s. 337.

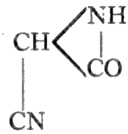
and the three nitriles in column (iii) are also transformed in a similar way, into their respective anhydrides—



the nitrile of pyrollidin-carboxylic acid
or its isomeride

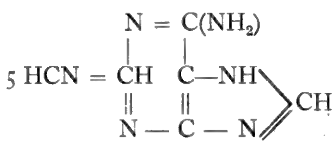


and



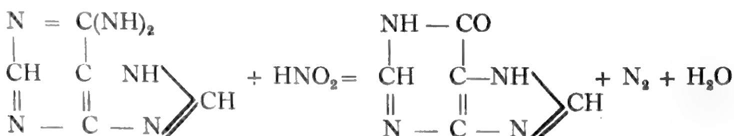
the last being the antecedent of di-amino propionic acid (see page 204).

As the proteid passes from the living to the dead state the amido-benzaldehyde in column (ii) is converted into the para-form, and the amino-malonic nitrile + 2 HCN is by molecular transformation converted into adenine (see page 203) a substance which according to Kossel[1] exists in all animal and vegetable cells. The composition of adenine is isomeric with prussic acid, its formula being $H_5C_5N_5$ or 5 HCN, and since by the action of nitrous acid it can be transformed into hypoxanthine, it belongs to the uric acid series and may be represented by the formula—



adenine

its transformation into hypoxanthine being represented by the following equation—



adenine hypoxanthine

1. *Zeitsch. f. physiol. Chemie*, Bd. X, s. 258.

Heated in a sealed tube with hydrochloric acid (sp. gr. 1·19) for from twelve to fourteen hours, adenine is resolved into glycocoll, formic acid, carbonic acid gas and ammonia,[1]

$$\underset{\text{adenine}}{H_5C_5N_5} + 8\,H_2O = \underset{\text{glycocoll}}{CH_2(NH_2)COOH} + \underset{\text{formic acid}}{2\,H \cdot COOH} + CO_2 + 4\,NH_3$$

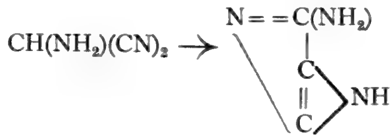
precisely what takes place when amino-malonic nitrile and two molecules of hydrocyanic acid are treated in the same way :—

$$\underset{\text{amino-malonic nitrile}}{CH(NH_2)(CN)_2} + 4\,H_2O = \underset{\text{glycocoll}}{CH_2(NH_2)COOH} + CO_2 + 2\,NH_3$$

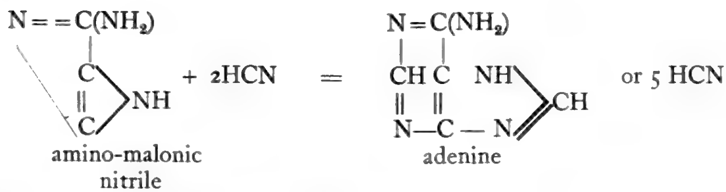
and

$$2\,HCN + 4\,H_2O = 2\,H \cdot COOH + 2\,NH_3.$$

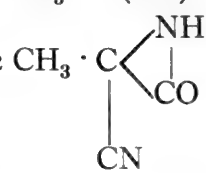
The relationship, therefore, between amino-malonic nitrile and adenine may be represented as follows :—



and



The compounds 3 HCN, 3 CNHS, $(CH_3 \cdot CO \cdot CN)_2$ in column (i) are transformed as the protein passes from the living to the dead state into 2 HCN, $(HCN)_2S_3$ and 2 $CH_3 \cdot C\,(OH) \cdot (CN)_2$, the latter being further transformed into

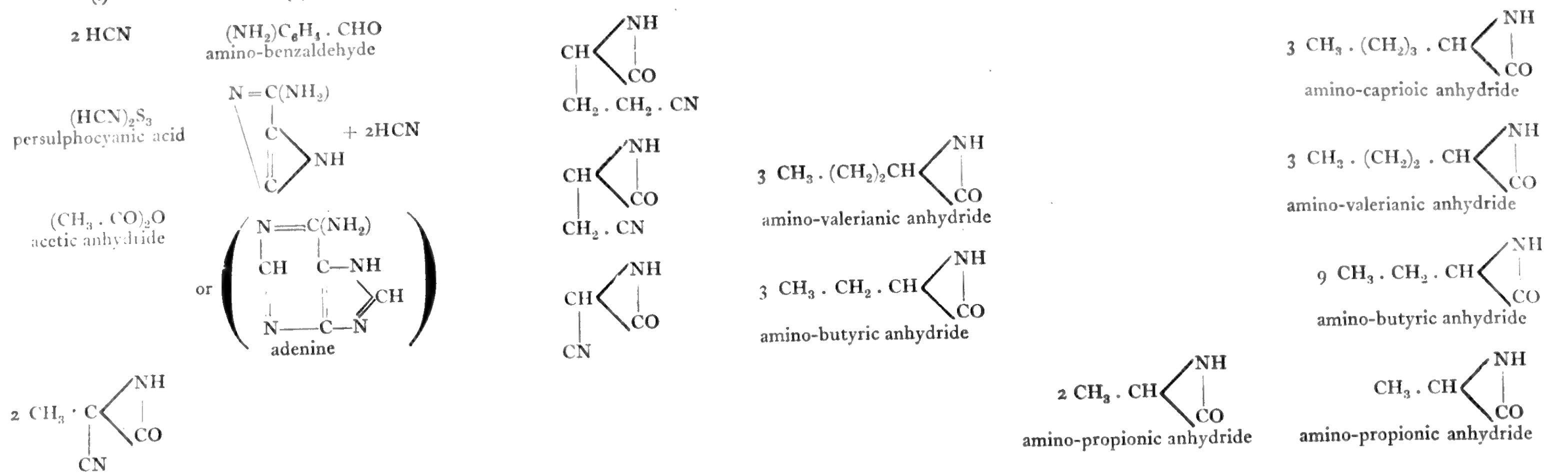


the antecedent of iso-malic acid (see page 201). The acetic anhydride remains unchanged. From the foregoing data the composition of dead albumin may therefore be represented in the following tabular form :—

1. Martin Krüger, *Zeitschrift für physiologische Chemie*, Bd. XVI, s. 160.

COMPOSITION OF DEAD ALBUMIN

(i)	(ii)	(iii)	(iv)	(v)	(vi)



(i)	(ii)	(iii)	(iv)	(v)	(vi)
		CH$_3$. C(NH) . CH(C$_2$H$_5$) . COOH imino-ethyl-acetyl-acetic acid			3 CH$_3$. (NH) . CH(C$_2$H$_5$) . COOH imino-ethyl-acetyl-acetic acid
		CH$_3$. C(NH) . CH(CH$_3$) . COOH imino-methyl-acetyl-acetic acid			3 CH$_3$. C(NH) . CH(CH$_3$) . COOH imino-methyl-acetyl-acetic acid
		CH$_3$: C(NH) . CH$_2$. COO imino-acetyl-acetic acid		2 CH$_3$. C(NH) . CH$_2$. COOH imino-acetyl-acetic acid	9 CH$_3$. C(NH) . CH$_2$. COOH imino-acetyl-acetic acid
					CH$_3$. C(NH) . COOH imino-pyruvic acid
$C_{16}H_{18}N_8O_5S_3$	$C_{12}H_{12}N_6O$	$C_{27}H_{39}N_9O_9$	$C_{27}H_{48}N_6O_6$	$C_{14}H_{24}N_4O_6$	$C_{144}H_{256}N_{32}O_{48} = C_{240}H_{397}N_{65}O_{75}S_3$
$+ 19\ H_2O$	$+ 8\ H_2O$	$+ 9\ H_2O$	$+ 6\ H_2O$	$+ 2\ H_2O$	$+ 16\ H_2O \quad = \quad + 60\ H_2O$
$C_{16}H_{56}N_8O_{24}S_3$	$C_{12}H_{28}N_6O_9$	$C_{27}H_{57}N_9O_{19}$	$C_{27}H_{60}N_6O_{12}$	$C_{14}H_{28}N_4O_8$	$C_{144}H_{288}N_{32}O_{64} = C_{240}H_{517}N_{65}O_{75}S_3$

Note Added June 5th, 1908

The four imino-ketonic acids—imino-pyruvic, -acetyl-acetic, -methyl-acetyl-acetic, and -ethyl-acetyl-acetic—which in the preceding tables are indicated as being constituents of protein, represent, in my opinion, the simplest forms of those bodies. If, however, imino-methyl-acetyl-acetic, and imino-ethyl-acetyl-acetic acids in either columns (iii) or (vi), or in both, were replaced by imino-laevulinic acid $CH_3 \cdot C\,(NH) \cdot CH_2 \cdot CH_2 \cdot COOH$ and imino-acetyl-butyric acid $CH_3 \cdot C\,(NH) \cdot (CH_2)_3 \cdot COOH$ (the formation of which is described on page 224) a different protein compound would result, having, however, the same molecular weight and the same ultimate composition as that given in the previous tables. Again, if for the amino-fatty acids in the tables, the iso-amino acids (the preparation of which from the ketones is in some degree indicated on page 244) were substituted, other protein substances would result; having, likewise, the same molecular weight and ultimate composition. These facts appear to me to explain why so many protein substances have the same ultimate composition but vary considerably both as to their physical and chemical properties.

THE OSMOTIC CONCENTRATION OF THE BLOOD OF FISHES TAKEN FROM SEA-WATER OF NATURALLY VARYING CONCENTRATION

By W. J. DAKIN, M.Sc., '51 *Exhibition Scholar, University of Liverpool.*

(*Received May 16th*, 1908)

It is now thirty-seven years since Bert (1), who was one of the first to consider the osmotic relations existing between the internal fluids of the animal body and the external fluids bathing their bodies, published a paper on the causes of death when freshwater fishes are plunged into sea-water. During the interval a great advance has taken place in physical chemistry, particularly with regard to the application of this branch of science to physiology and medicine, and numerous observers have turned their attention to the constitution and physical properties of the 'internal' and 'external media' both for invertebrates and vertebrates. Fredericq (4) published in 1885 an account of some investigations concerning the relation of the salt contents of the blood of Crustacea to the salt contents of the sea or fresh water in which the animals were living, and from that date to the present time the osmotic conditions of the blood and other body fluids together with the nature of the bounding membranes have been studied either by chemically estimating the constitution of the internal and external media or by determining directly the osmotic pressure with the aid of the Beckmann's freezing point apparatus.

It is not necessary in this introductory communication to go into the history of the discoveries made in this line of research, but it may be mentioned here that various problems which are linked together and stand in very close relation to the constitution of the internal and external media and to the bounding membranes have been considered from several points of view. For example, the relation existing between the sea-water and the blood and coelomic fluids of marine invertebrates has been investigated from the purely

physico-chemical point of view, and again, the physiological action of various fluids, such as sea-water, on freshwater animals, and fresh water on marine animals with regard to duration of life in these media, has been considered. The results of the various experiments made by different observers, often differ considerably, as do naturally the theories deduced from these results. Fredericq (5), Rodier (12), Quinton (11), Garrey (6) and others have shewn that the blood and fluids of the body cavity, coelom, or haemocoele, of invertebrates have practically the same osmotic pressure as the water in which they live, and contain almost the same percentage of salts in solution. Moreover, as the sea-water in the case of marine invertebrates varies in salt contents and osmotic pressure, so do the fluids of the body change accordingly. Turning now on the other hand to the vertebrates, we find in the highest vertebrates, the mammals, a *constant* or *practically constant* osmotic pressure for the blood, amounting to about seven atmospheres, and this is held in defiance of alterations in the constitution of the food. For other higher vertebrates this also holds, and though the Amphibia have a somewhat lower osmotic pressure corresponding to a 0·7 per cent. salt solution, Overton (10) has demonstrated the action of the organism in keeping this pressure constant.

One finds, however, on investigating the same question in the fishes that there is a surprising difference. The blood of Elasmobranchs possesses about the same osmotic pressure as the external medium, the sea-water, and further, this pressure is not constant but, as is the case for the invertebrates, varies with changes in the external medium. Teleosts, on the other hand, are quite different, and appear to resemble more the higher vertebrates in keeping a constant osmotic pressure. The freezing point depression of the teleost blood appears from Garrey's work to average about 0·872°, though it is subject to slight variations. The surprising feature here is that in the teleostei, though the blood is brought into close connection with the sea-water by the gills, the fish contrives to maintain an osmotic pressure which is only about one-third of that of the surrounding sea-water. It should be mentioned also that the Elasmobranchs, though resembling the invertebrates in having a varying osmotic pressure for the blood which

is almost the same as that of the external medium, have a salt contents much less, resembling in fact the proportion of salts in the blood of the teleosts. The osmotic pressure, however, is brought up by the presence of considerable quantities of urea in the blood. We have therefore, roughly speaking, the following three groups of animals, members of which live in water but do not breathe air directly :—

1. *Invertebrates.*—Osmotic pressure and salts contents of blood and internal media practically identical with external medium.
2. *Elasmobranchs.*—Osmotic pressure of blood practically identical with external medium, but salts contents much lower.
3. *Teleosts.*—Osmotic pressure and salts contents of blood much lower than that of external medium.

Thus it appears as if the independence of the constitution of blood is first established in the teleosts. What determines this difference ? Is the membrane, either gills or body wall, of the invertebrates so different in constitution from that of the teleosts that in the first case perfect osmotic conditions are set up, and the internal fluids are directly dependent on the external, whilst in the latter the membranes are absolutely impermeable to the external medium ?

For invertebrates Fredericq and Quinton state that the bounding membranes are permeable to both water and salts, whilst Botazzi and Enriques (2) state that the membranes are semi-permeable, that is, they allow water to pass through and, therefore, bring about the osmotic equilibrium but are impermeable to salts.

For teleosts, Dekhuyzen (3) states from a series of observations made at Bergen, that they have a definite osmotic pressure of the blood, and that any differences occurring are probably due to differences in pathological conditions or variations in observation. Sumner (13) has, however, quite recently conducted an extensive series of experiments for the purpose of investigating the conditions of the external membranes in teleosts, the fish used being chiefly three species of Fundulus, small fishes which pass into brackish waters and one species of which occurs frequently in fresh water. By

weight determinations before and after placing a number of these fishes directly or gradually into fresh water or into sea-water diluted considerably with fresh water, he found that a considerable increase or decrease in the weight of the fishes resulted from changes in the salinity and hence osmotic pressure of the external medium, and came to the conclusion that though the osmotic condition of the blood and the external fluids in the teleostei are so different, yet the membrane of the gills is both permeable to water and probably to a smaller extent to salts. This is opposed to the views of Fredericq (4) who states that the membranes of teleosts are effective barriers to the external medium, and to Garrey who also supposes an impermeability. Fredericq (4) makes, moreover, the statement that the blood of salt-water fishes does not taste or tastes scarcely more salt than that of freshwater fishes, and that the muscles and glands of a salt-water fish contain no more salt than that of a freshwater one. Griffiths (8) also states that the blood of a sole or haddock does not contain more soluble salts than that from freshwater fishes. With regard, however, to these estimations of chlorine and salts, there seems to be some confusion. Atwater gives figures which are 15 per cent. lower for chlorine in the constitution of freshwater fishes than in marine, his average being 0·235 per cent. Cl for marine fishes, Quinton (11) gives the chlorine percentage of the blood of eight species of marine teleosts as 0·651 and freshwater teleosts as 0·411. This, as Sumner (13) points out, is much greater than the chlorine contents of the body as a whole, and also shews that the salinity figures for marine fishes are almost 50 per cent. greater than for freshwater fishes. Sumner has, unfortunately, not given the osmotic pressures as determined directly by the freezing point method for the blood of the fishes in which the changes in weight occurred in his experiments. If the gills are semi-permeable, that is only permeable to water, or to a small extent for salts in addition to this, then the osmotic pressure and chlorine contents of the blood of freshwater fishes should be lower than that of marine fishes, and this change should be found to take place in those teleosts which pass from the one medium to the other, like the eel and salmon. Garrey states that transferring common eels

from salt water to fresh water did not lower the osmotic pressure of the blood. Greene (7), on the Physiology of the Chinook Salmon, finds a lower osmotic pressure in those caught in fresh water, but thinks the small difference may be due to absence of food or to changes in the metabolism due to the changes in habits and the breeding period.

In view of these conflicting results I was encouraged to take up this investigation through a suggestion of Prof. Brandt at Kiel, and to examine the osmotic pressures and chlorine contents of the blood of fishes living under natural conditions and not alone under the experimental aquarium conditions. This is in many cases a matter of great difficulty, and most of the experiments previously made, including Sumner's, have been performed under the somewhat artificial conditions of the aquarium. It seemed very important, therefore, to supplement the aquarium results, and I was fortunately able to do so by obtaining permission to take my apparatus on the German investigation steamer 'Poseidon' on one of the expeditions from Kiel through the Kattegat and Skagerack to the North Sea. In this way I was able to examine fish from water of gradually varying density, and the number was only limited by the very bad weather this last February rendering it often both impossible to trawl for the fish and to perform any other experiments.

The first determinations were made at Kiel University, where fish were easily obtained living, since they are brought into the harbour at Kiel in submerged boxes and kept in the water until the actual moment of selling. The osmotic pressure has been determined by the freezing point method, and in order to make more certain of the correctness of the results, the freezing point of distilled water was determined before and after each series of observations. The Beckmann thermometer differed slightly from those in general use in having a shorter bulb so that only 10 to 15 c.c. of blood were necessary instead of the 18 to 20 c.c. usually required. This I found to be a most important acquisition, since it is often difficult to obtain larger quantities of blood from small specimens. The thermometer was made by Goetz, Leipzig. In almost all cases the blood was obtained by cutting the caudal artery and withdrawing the blood with a pipette.

It was always easy to obtain blood in this way, if the fish was actually living at the time and the heart beating. This occurred in every case, so that the blood was taken from the living fish and the osmotic pressure determined immediately, allowing no errors to creep into the results from decomposition. It has been stated by Hamburger (14) and Hedin (15) that it is the same whether one uses the blood, blood plasma, or serum, because the blood corpuscles in suspension have as little effect on the osmotic pressure as sand grains, but no decomposition should have taken place. Further investigations are being made with regard to this point but need not be considered here since for purposes of comparison the blood for the following experiments was always taken in the same way and the whole blood was used in every case.

The *depression* of the freezing point is expressed here in the usual way as Δ and the comparisons are made in terms of this depression. The actual pressure in atmospheres can be found by multiplying the depression in degrees by $\frac{22 \cdot 4}{1 \cdot 85}$. After the determination of the Δ, the blood was removed from the Beckmann's apparatus, and a quantity carefully weighed and transferred to a porcelain crucible. Powdered chlorine-free sodium carbonate was added and the whole evaporated to dryness and then slowly incinerated by moderate heat to prevent any considerable loss of chlorine. The residue was extracted with hot distilled water, filtered, and determined by Volhard's method. To the filtrate nitric acid was added in slight excess. To this solution a definite quantity of $\frac{N}{10}$ $Ag\ NO_3$ was added from a burette, taking care to use more than required to precipitate the chlorine so that in the solution there is an excess of silver nitrate. The precipitated silver chloride was filtered off, and the amount of silver nitrate in the filtrate determined by titrating with potassium sulphocyanide, using iron ammonium alum as indicator. This gives the quantity of excess, and by subtracting it from the quantity originally taken one has the amount of standard silver equivalent to the chlorine in the solution, from which the chlorine percentage can be easily reckoned out.

The experiments made have been grouped into series according to time and place, beginning at Kiel and ending at Helgoland. In every case where the Δ is given, this is the average of three determinations, the degree of 'under cooling' being kept small and, as far as possible, the same throughout.

Series I—Feb. 5th, 1908, *Kiel Harbour* :—

Sea water from Kiel Harbour Δ − 1·093°

Chlorine contents of harbour water 1·125 per cent.

Salt contents 2·033 per cent.

Blood from cod (*Gadus morrhua*) Δ − 0·720°

Chlorine contents of blood 0·50 per cent.

Series II, Feb. 6th, Kiel Harbour :—

Blood from *Gadus morrhua* Δ − 0·750°

Blood from *Gadus morrhua* Δ − 0·751

Chlorine contents of blood 0·50 per cent. and 0·503 per cent. respectively.

Series III, Feb. 10th, Kiel Harbour :—Blood taken from three specimens of *Pleuronectes platessa* (plaice), a very small quantity of blood is obtainable from a single fish.

Depression of the freezing point Δ = − 0·66°

Chlorine contents of the same blood 0·500 per cent.

The difference in the osmotic pressure between the blood of the cod and the plaice is here notable, and illustrates the differences which are found to occur amongst different species of teleosts from the same water.

Series IV, Feb. 12th, Kiel Harbour :—Blood from three large *Pleuronectes platessa* taken as in the previous experiment—

Depression of the freezing point Δ = − 0·650°

Chlorine contents of the same blood 0·531 per cent.

The blood for both determinations was a mixture of that from the three fishes used.

Depression of the freezing point for ovarial fluid from the same fishes Δ = − 0·630.

Series V, Feb. 16th, 1908—*On S.S.* '*Poseidon*.'—Baltic Sea, just outside the Kieler Bucht. Depth 27 metres. Temperature at bottom 1·88° C. Salts contents of bottom water 2·6 per cent., and of surface water 1·46 per cent.

Depression of the freezing point for bottom water Δ − 1·3°

The following determinations were made :—

Gadus morrhua Δ − 0·758°

Gadus morrhua Δ − 0·710

Gadus morrhua Δ − 0·730

For each of the following determinations, three fish were used :—

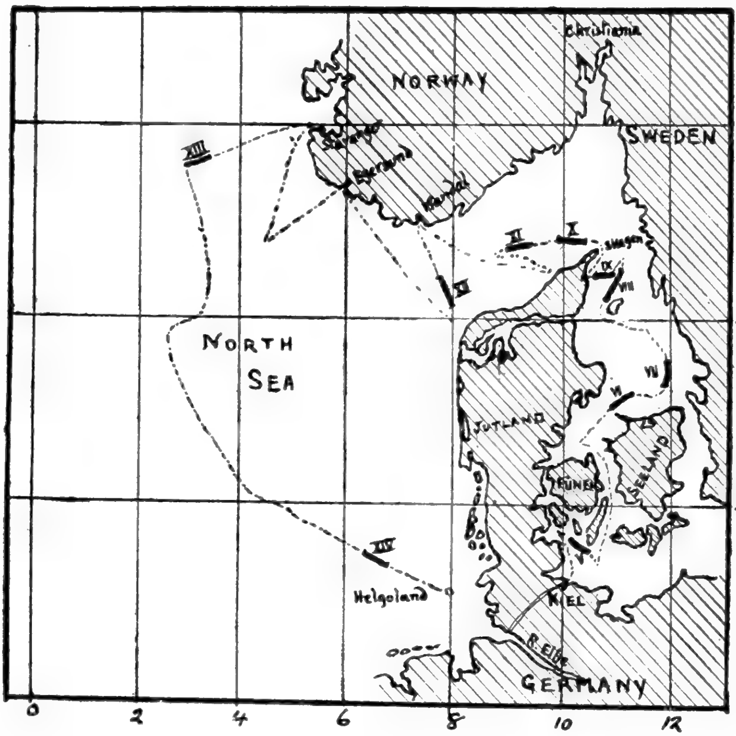
Pleuronectes platessa Δ = − 0·718°

Pleuronectes platessa Δ = − 0·720

Series VI, Feb. 17th, 1908, *S.S. 'Poseidon.'* — Station, Kattegat III. Depth 21-24 metres. Between Jutland and Seeland. Temperature at bottom 2·99° C. Sp. gr. at bottom[1] 24·6. Salt contents: surface 2·93 per cent., and bottom 2·97 per cent.

A sample of bottom water gave ...	$\Delta = -1{\cdot}665°$
Pleuronectes flesus (2 specimens used) ...	$\Delta = -0{\cdot}96$
Gadus morrhua	$\Delta = -0{\cdot}715$
Pleuronectes platessa (4 specimens used) ...	$\Delta = -0{\cdot}73$
Raia radiata	$\Delta = -1{\cdot}51$

This last named was the first elasmobranch examined, the great difference from the teleosts and the resemblance to the Δ for sea-water is noticeable.



CRUISE OF 'POSEIDON,' Feb.-March, 1908.—The stretches in black connected by the dotted line indicate the places where the trawl was used, and the numbers the series of determinations referred to in the text.

1. The figure given above (and in the succeeding determination for the Specific Gravity of the sea water) + 1000 equals the true Specific Gravity, that of distilled water being considered as 1000.

Series VII, Feb. 17th, S.S. 'Poseidon.'—Kattegat, near coast of Sweden and about thirty miles North of Seeland. 37-57 metres deep. Temperature at bottom 3·7° C. Sp. gr. at bottom 26·0. Salt contents : surface 1·93 per cent., bottom 3·15 per cent.

	A sample of bottom water gave	...	$\Delta = -1{\cdot}71^\circ$
1.	*Gadus morrhua*	...	$\Delta = -0{\cdot}8$
2.	*Gadus morrhua*	...	$\Delta = -0{\cdot}77$
	Gadus aeglefinus	...	$\Delta = -0{\cdot}75$
	Raia batis	...	$\Delta = -1{\cdot}820$
1.	*Acanthias vulgaris*	...	$\Delta = -1{\cdot}820$
2.	*Acanthias vulgaris*	...	$\Delta = -1{\cdot}795$

For the determination of the blood from *Gadus aeglefinus*, five or six fish were used. All the remaining fish were large, the cod being 1·3 metres in length. The increase in the depression of the freezing point for elasmobranch blood is to be noticed here, and the likeness between the two species.

Series VIII, Feb. 18th, S.S. 'Poseidon.'—Kattegat, between Denmark and Sweden, and direct East of Frederikshavn. 24-40 metres deep. Temperature at bottom 4·9° C. Salt contents : surface 3·447 per cent., bottom 3·445 per cent. Sp. gr. : surface, 28·2, bottom 28·2.

	A sample of bottom water gave	...	$\Delta = -1{\cdot}86^\circ$
1.	*Gadus morrhua*	...	$\Delta = -0{\cdot}75$
2.	*Gadus morrhua*	...	$\Delta = -0{\cdot}76$
	Rhombus laevis	...	$\Delta = -0{\cdot}071$

Series IX, Feb. 19th, S.S. 'Poseidon.'—In Kattegat, direct East of and not far from Frederikshavn, Denmark. Conditions practically the same as above, both catches were trawled in the same stretch of water and no further observations taken.

Rhombus maximus	...	$\Delta = -0{\cdot}79^\circ$

Series X, Feb. 20th.—In Skagerack, West of and not far from Skager Point. Depth 92 metres. Temperature at bottom 5·02° C. Sp. gr., surface 28·4, bottom, 28·4. Salt contents, surface and bottom, 3·472 per cent.

	A sample of bottom water gave	...	$\Delta = -1{\cdot}893^\circ$
	Gadus morrhua	...	$\Delta = -0{\cdot}74$
1.	*Anarrhichas lupus*	...	$\Delta = -0{\cdot}84$
	Anarrhichas lupus	...	$\Delta = -0{\cdot}73$
	Cyclopterus lumpus	...	$\Delta = -0{\cdot}66$

The *Anarrhicas* were large specimens, length respectively 78 and 71 centimetres. The rather remarkable difference between these two fishes with regard to the osmotic pressure is another case of the variation sometimes noticed for the same species, in the same water. The other interesting feature here was the low osmotic pressure of the *Cyclopterus* blood.

Series XI, S.S. 'Poseidon,' Feb. 21.—Skagerack, middle of channel, between Norway and Denmark. Depth 130-192 metres. Bottom temperature 6·76° C. Sp. gr: surface 28·1, bottom 28·6. Salt contents: surface 3·434 per cent., bottom 3·519 per cent.

Freezing point of the bottom water	...	$\Delta = -1{\cdot}92°$
Lophius piscatorius	...	$\Delta = -0{\cdot}63$
Gadus aeglefinus	...	$\Delta = -0{\cdot}74$

The *Lophius* weighed 14 kilos and was, therefore, of considerable size; the blood was easily obtained, and the extremely low osmotic pressure is rather remarkable for a marine fish.

Series XII, S.S. 'Poseidon,' Feb. 26th.—Middle of channel on the boundary of Skagerack and North Sea. Depth 54 metres. Bottom temperature 5·31° C. Sp. gr.: surface 28·55, and bottom 28·65. Salt contents: surface 3·499 per cent., bottom 3·511 per cent.

A sample of bottom water gave	...	$\Delta = -1{\cdot}96°$
Anarrhichas lupus	...	$\Delta = -0{\cdot}74$
Lota molva	...	$\Delta = -0{\cdot}66$
Hypoglossus vulgaris	...	$\Delta = -0{\cdot}78$
Lophius piscatorius	...	$\Delta = -0{\cdot}68$
Gadus pollachius	...	$\Delta = -0{\cdot}73$
Raia valonia	...	$\Delta = -2{\cdot}0$

The fish used for the above determinations were all of very large size, the *Lota molva* being 131 centimetres long and weighed 17·5 kilos.

Series XIII, S.S. 'Poseidon,' March 5th.—North Sea about 100 miles West of Stavanger, Norway. Depth 105 metres. Bottom temperature 6·78°. Sp. gr.: surface 28·2, and bottom 28·6. Salt contents: surface 3·467 per cent., and bottom 3·512 per cent.

Bottom water	...	$\Delta = -1{\cdot}95°$
Gadus morrhua	...	$\Delta = -0{\cdot}70$
Gadus virens	...	$\Delta = -0{\cdot}71$
Gadus aeglefinus	...	$\Delta = -0{\cdot}78$
Lota molva	...	$\Delta = -0{\cdot}68$

The *Gadus morrhua* has a remarkably low osmotic pressure here, which is probably pathological; extremely few specimens were present in the trawl.

Series XIV, S.S. 'Poseidon,' March 7th.—North Sea, about 70 miles North-West of Helgoland. 40 metres deep. Bottom temperature 4·53° C. Sp. gr.: surface 28·5, bottom, 28·5. Salt contents: surface 3·479 per cent., and bottom 3·485 per cent.

	Bottom water	$\Delta = -1{\cdot}90°$
1.	*Gadus morrhua*	$\Delta = -0{\cdot}73$
2.	*Gadus morrhua*	$\Delta = -0{\cdot}79$
3.	*Gadus morrhua*	$\Delta = -0{\cdot}75$
4.	*Gadus morrhua*	$\Delta = -0{\cdot}77$
	Raia clavata	$\Delta = -1{\cdot}99$

With the exception of the Ray and the specimens of *Gadus morrhua*, the contents of the trawl were small plaice. The four specimens of *Gadus morrhua* were used to determine to what extent variations might occur in the same species caught in the same water.

Series XV, Helgoland, April, 1908.—

Blood from three specimens *Pleuronectes platessa* ...	$\Delta = -0{\cdot}78°$
" " three " " " ...	$\Delta = -0{\cdot}848$
" " five " " " ...	$\Delta = -0{\cdot}750$
" " three " " " ...	$\Delta = -0{\cdot}773$

Chlorine estimation for blood used in the second and third of the above determinations 0·60 per cent.

Chlorine estimation for blood used in the last determination 0·537 per cent.

Series XVI, Helgoland, May, 1908.—

Gadus morrhua—blood	$\Delta = -0{\cdot}748°$
Blood from two specimens *Gadus morrhua* ...	$\Delta = -0{\cdot}778$
Blood from five specimens *Gadus morrhua* ...	$\Delta = -0{\cdot}748$

Chlorine estimation for blood from the first specimen 0·507 per cent.

Chlorine estimation for blood from the cod used in the last two determinations 0·530 per cent.

Freezing point for bottom water $\Delta = -1{\cdot}90$.

Salt contents of bottom water 3·485 per cent.

The first point to be noticed in this series of estimations, is the great variation that occurs both amongst different specimens of the same species as well as between the different species themselves. It shews the great necessity of making a number of determinations before deducing any theories. For the teleosts examined the Δ varied from $-0{\cdot}63$ to $-0{\cdot}96$ and this was not due to any great difference in the external medium since the fishes with the average $-0{\cdot}96$ came from water with a lower Δ than the fish with Δ 0·63. It was therefore obvious that the different species could not be compared directly together unless specimens of the same species had been caught at all

or at many stations. Now the most important determinations are those made at Kiel and Helgoland where the convenience of a laboratory was at hand, and unfortunately only two species could be obtained living at Kiel, namely *Gadus morrhua* and *Pleuronectes platessa*. Fortunately, however, the codfish were caught at almost every station, and so form an interesting series; and plaice were also caught in Helgoland to allow of a comparison with the Baltic Sea specimens. The following table gives the determinations made on the plaice :—

Place where fish were caught	Δ for blood	Δ for sea-water	Salt contents of sea-water per cent.	Chlorine in blood per cent.
Kiel	− 0·66 − 0·65	− 1·093	2·032	0·500 0·531
Series V.—In Baltic Sea ...	− 0·718 − 0·72	− 1·3	2·60	—
Series VI.—Kattegat ...	− 0·73	− 1·66	2·97	—
Helgoland	− 0·78 − 0·848 − 0·750 − 0·773	− 1·90	3·48	0·60 0·537

It will be seen that a direct increase in the osmotic pressure of the blood takes place as the density and osmotic pressure of the sea-water increases. The average for Kiel is − 0·655, and for Helgoland − 0·787; that is, whilst the sea-water bathing the fish has increased in osmotic pressure so that the Δ has changed from − 1·093 to − 1·90, an increase of 74 per cent. or almost 10 atmospheres, the osmotic pressure of the blood has increased by 20·1 per cent. or about 1·5 atmospheres.

This is a very interesting result and shows that the plaice at least have an osmotic pressure which is dependent to a certain extent on the sea-water, though nothing like the Elasmobranchs, since an increase of 74 per cent. in that of the sea-water produces an increase of but 20·1 per cent. in the fish. The chlorine contents also shew an increase corresponding to the increase in the osmotic pressure; the average for Kiel being 0·515 per cent., and for Helgoland 0·557 per cent. The relation of these changes will be discussed after the results for the cod are given in tabulated form.

Results of Chlorine and Osmotic Pressure Determinations for the Cod (Gadus morrhua)

Place where fish were caught	Δ for blood	Δ for sea-water	Salt contents of sea-water per cent.	Chlorine in blood per cent.
Kiel	− 0·720			0·50
	− 0·750	− 1·093	2·033	0·503
	− 0·751			0·50
Series V.—In Baltic Sea ...	− 0·758			
	− 0·710	− 1·30	2·6	—
	− 0·730			
Series VI.—In Kattegat ...	− 0·715	− 1·66	2·97	—
Series VII.—In Kattegat ...	− 0·80	− 1·71	3·156	—
	− 0·77			
Series VIII.—In Kattegat	− 0·75			
	− 0·76	− 1·86	3·444	—
Series IX.—In Skagerack ...	− 0·74	− 1·893	3·472	—
Series XIII.—North Sea ...	− 0·70	− 1·95	3·512	—
Series XIV.—North Sea ...	− 0·73			
	− 0·79			
	− 0·75	− 1·90	3·485	—
	− 0·77			
Helgoland	− 0·748	− 1·90	3·485	0·507
	− 0·778			
	− 0·748			0·530

The figures in the above table form a rather startling contrast to those for the plaice. More specimens have been used for the estimations of codfish blood than that of any other species, perhaps forty or more in all, and never are two alike. There seems to be a very considerable variation in specimens of the cod taken from the same place, considerable when compared with other teleosts that I have examined since, and this variation is greater than the actual change of the blood between the two places Kiel and Helgoland.

If the average be taken for the first two series Kiel and Baltic Sea specimens the Δ is − 0·73 ; the fish are from water of Δ − 1·093 and Δ − 1·30. In the same way the average for the fishes from Series VI, VII and VIII is − 0·759, the sea-water being Δ − 1·66—Δ − 1·86, and lastly Series IX—Helgoland gives an average Δ − 0·757 (leaving out the fish with Δ 0·70 caught in Series XIII and which

is probably in a pathological condition), with sea-water $\Delta - 1{\cdot}89$—Δ $1{\cdot}90$.

Thus if the averages of these three sets are taken, there is a slight increase between the fishes of the Kiel and Baltic Sea examined, and those of the Kattegat, Skagerack and North Sea, but instead of an increase of 20 per cent. as occurs with the plaice, there is only an increase of about 3·9 per cent. in the blood for an increase of 74 per cent. in the sea-water. I mention these averages in order to shew that if any steady variation can be said to take place it is in the direction of an increase in the denser water of the North Sea. The difference in the averages of the Kiel and Helgoland fishes taken separately from the others is as follows:—Kiel average $\Delta = -0{\cdot}740$, Helgoland $\Delta - 0{\cdot}758$—an increase of $\Delta - 0{\cdot}018$, whilst the variation amongst specimens caught at the same place is in one case $\Delta - 0{\cdot}73$—$\Delta - 0{\cdot}79$.

The conclusion arrived at therefore here, is that a slight increase of osmotic pressure occurs in the blood of cod caught in water of higher salt contents, as the North Sea, but that this increase is only small compared with that taking place in *Pleuronectes platessa* under the same conditions, and is furthermore overshadowed by individual variation.

Another point to be noticed in the series of experiments made on the 'Poseidon,' and which has been verified by further observations made at Helgoland, is the differences occurring between the species. The numbers given by previous observers, often from single observations, have not sufficiently emphasised these differences, since they may be put down in the same category as those occurring between individuals of the same species. If, however, averages are taken for different teleosts, it will be found that in spite of variation there is a 'mean' for each species which is not the same. Thus, for example, *Lota molva* and *Lophius piscatorius* have a low osmotic pressure average $\Delta = -0{\cdot}65$, whilst that for *Pleuronectes flesus* is much higher averaging about $\Delta = -0{\cdot}85$, for fish caught in sea-water of $\Delta - 1{\cdot}90$.

The figures for the Elasmobranchs agree very satisfactorily with what was expected from the results of Botazzi; there is little variation between different species caught in the same water, the

osmotic pressure is almost the same as that of the surrounding sea-water, and as this increases in density so does the osmotic pressure of the blood change. Thus the first Elasmobranch caught on the voyage, a specimen of *Raia radiata*, gave $\Delta - 1{\cdot}51$, the Δ for the sea-water being $- 1{\cdot}66$, and later when the osmotic pressure of the sea-water had increased and the Δ was $- 1{\cdot}95$— $- 1{\cdot}98$, two specimens, *R. valonia* and *clavata*, gave for the blood $\Delta - 2{\cdot}0$ and $- 1{\cdot}99$ respectively.

In view of the small amount of change taking place in the blood of the cod and on account of the above experiments generally, it was thought advisable to determine the osmotic pressure and chlorine contents of some freshwater teleosts that were obtainable in Kiel, and particularly for the eel, in order to see what difference existed in the blood of typical freshwater fishes. The specimens had all been kept in fresh water in large well-aerated tanks for a few days from the time that they had been removed from the breeding ponds and freshwater lakes in Schleswig Holstein in the neighbourhood of Kiel.

Tank water in which fishes were living ...	...	$\Delta - 0{\cdot}020°$
Carp	blood	$\Delta - 0{\cdot}487$
Abramis brama	„	$\Delta - 0{\cdot}510$
Eel (*Anguilla vulgaris*)	„	$\Delta - 0{\cdot}570$

The chlorine estimations for the blood of the above fishes gave the following results :—

Carp	blood contained... ...	0·2 per cent. Cl
Abramis brama	do.	0·253 „ „
Eel	do.	0·277 „ „

Thus the osmotic pressure and chlorine contents of the blood of freshwater fishes is much below that of marine teleosts, and the statements of Griffiths and Fredericq with regard to the salt contents of freshwater fishes cannot be regarded as correct, for the marine teleosts have about 50 per cent. greater chlorine and, therefore, salts contents. These figures agree with those of Quinton's for other freshwater teleosts, and it must be observed that the chlorine contents of the blood is much higher than the chlorine contents of the body, or the whole fish.

These determinations were completed by investigating the

changes taking place in the blood of the freshwater eel when placed in sea-water, and I am at present engaged on the complementary experiments with the same species of eel caught in the sea. A large eel taken from the freshwater tank was placed abruptly into slightly diluted North Sea water, the Δ for which was − 1·59. This abrupt change was followed by a copious secretion of mucous, but after a few hours the specimen appeared quite normal. After an interval of six hours in this water the blood was taken and examined.

Blood	... ...	Δ − 0·66°
Chlorine	... ...	0·367 per cent.

There is evidence here of considerable change, for, compared with the Δ for the eel in fresh water, the freezing point is 16 per cent. lower and the chlorine contents have also increased considerably.

Another still larger specimen was therefore taken, as before, directly from fresh water and placed this time in sea-water rather more diluted with fresh (sp. gr. 20·3). This specimen endured the change more calmly and without such a mucous secretion. The following was the procedure :—

10.30 a.m.—Eel placed abruptly from fresh water into diluted sea-water, sp. gr. 20·3.
3.45 p.m.—Sp. gr. of water increased to 25·5 by addition of North Sea water.
6.45 p.m.—Sp. gr. of water, further increased to 29·3 by addition of some concentrated sea-water.

The eel remained overnight in this water and at 10.30 a.m. the next morning, after a sojourn of twenty-four hours in sea-water, the eel being quite healthy in appearance, the osmotic pressure of the blood was determined.

Blood from eel after 24 hours in sea-water ... ... Δ = − 0·745°

Hence the osmotic pressure had increased until it attained a pressure which is about an average for marine teleosts.

The osmotic pressure of the Teleostei in fresh water averages about Δ − 0·527, and for those in North Sea water the Δ is about − 0·750, hence for those fishes like the eel and salmon which pass from the sea to the rivers there must be a place where the external water is isotonic with the blood. These fishes are, in short, capable

of passing from a hypertonic fluid to a hypotonic fluid without injurious effects following. Of the marine teleosts which do not pass far into the rivers there are many which are found in the Baltic Sea, far East of Kiel, for example, *Cottus scorpius*, *Pleuronectes flesus*, *Gadus morrhua*, *Lota vulgaris*, *Nerophis ophidion*, occurring frequently together with others only occurring as visitors. Whether these species that occur frequently have passed their whole lives in this area is a question. In all probability they do pass their whole lives and lay their eggs in this region. Now it will be seen from hydrographical tables that in the North-East Baltic where the fishes named above occur, the water has a salt contents below that of the fishes' blood and is hypotonic. Thus marine teleosts are often found in waters isotonic and hypotonic to the blood. The Elasmobranchs on the other hand are only rare and as visitors in the West Baltic, and altogether absent from the South-East and North-East Baltic. This appears to be correlated with the fact that the constitution and osmotic pressure of the blood is much more dependent on the osmotic pressure of the external media than is the case in the Teleostei, but further experiments on the resistance of teleosts and elasmobranchs to changes in the constitution of the sea-water are being made and the results will be discussed later.

Conclusion and Summary

It has been shewn by previous observers that the marine teleosts have an osmotic pressure and salt contents differing to a great extent from that of the sea-water bathing their bodies, and in this respect shew a remarkable contrast to both the marine elasmobranchs and invertebrates. Certain workers have assumed from this that the body membranes separating the blood and internal fluids from the sea-water were impermeable barriers to water and salts. Sumner, by weight and chlorine analyses came to the conclusion that this was not the case, and that to a certain extent both water and salts could pass through these bounding membranes.

Now we may assume here four possibilities for the bounding membranes :—

1. The membranes are impermeable to water and to salts.
2. The membranes are semi-permeable, allowing water to pass through but impermeable to the dissolved salts.
3. The membranes are permeable to water and in a slight degree to salts.
4. The membranes are permeable to both water and salts.

If the first of these possibilities held good, it would be easy to understand how the teleosts retained their low osmotic pressure and salt contents, against the influence of the sea-water, but no change either in osmotic pressure or weight should then take place if the animals are placed in different media. This change does take place, for the experiments shew that in natural conditions the freshwater teleosts have a lower osmotic pressure than the marine forms, that this changes with the alterations in the water (as for example in experiments with the eel), and that even alterations in the salt contents prevailing in the sea, influences the osmotic pressure of the blood. Thus we may consider the first possibility, of the teleosts having impermeable membranes, as quite disproved.

If the fourth possibility held good and the membranes were directly permeable to both water and salts, then the conditions for an osmotic change would not prevail and no change in weight should occur if the fishes were taken from salt-water and placed in fresh water. Sumner has shewn, however, that a change does occur and that water passes osmotically into the fish, increasing the weight. Moreover, if this was the case it would be difficult to conceive of the alterations in the osmotic pressure of the external media—for example the difference between that of the Baltic Sea water and the North Sea water, or the fresh and salt water in the case of the eel experiments—producing such a little change in the constitution of the blood. Furthermore, it would mean that some organ or organs were continually at work to such an extent that the osmotic pressure and salt contents of the marine teleosts were kept regularly at about one-third of that of the water bathing the outside of the membranes under consideration. This fourth possibility, is therefore, also impracticable, and we are reduced to the second and third.

If the limiting membranes of the body are semi-permeable, that is, permeable to water but not to salts, then perfect osmotic conditions are set up, and any such increase of the osmotic pressure on the outside of the membrane as takes place in passing from the Baltic Sea to the North Sea, would cause a corresponding increase in the osmotic pressure and chlorine contents of the blood, since more water would pass out from the blood and body fluids through the membranes into the sea and leave the blood and fluids more concentrated. Similarly, when placing an eel from fresh water into salt water, the great increase in osmotic pressure in the external medium should cause water to leave the blood and body fluids, making the concentration and osmotic pressure of these latter higher than before. This is exactly what takes place in the experiments. Sumner assumes also a slight permeability to salts, but only slight, so that the osmotic conditions are preserved and exactly the same state of things would occur. Hence we must conclude that the bounding membranes of the teleostei are either semi-permeable, permeable for water and not for salts, or to a small extent for salts. I am of opinion at present that the permeability is only for water, and that Sumner's experiments do not indicate conclusively a permeability for salts ; this, however, will be discussed in a later paper. The increase in chlorine contents in these experiments does not prove that chlorine has passed into the blood, since the same result would be obtained by water passing out, and therefore increasing the concentration. If now we conclude that either of these two possibilities holds good, and the experiments prove this to be the case, then how do the marine teleosts contrive to maintain an osmotic pressure and salt contents much lower than that of the external medium, whilst freshwater teleosts have a higher osmotic pressure and salt contents than the external water ?

Allowing that the membranes are permeable to water, then there must be a continual stream of this fluid from the bodies of marine teleosts outwards into the sea-water and *vice versa* in the freshwater teleosts, but in spite of this the constitution of the blood remains practically constant. I believe this to be accounted for by the three following assumptions :—

1. The permeability for water is not very great.
2. The permeable membranes are of small extent.
3. The actual loss or gain in water by the blood is counteracted by resorption and secretion.

The permeable membranes of the teleosts appear to be confined to the gills, the body walls of the fish being impermeable, because fresh water has practically no harmful effect on a marine teleost if the body part only is immersed in it, and sea-water flows over the gills. The permeability of the gills for water must not be great or one would have the secreting organs working continually at 'high pressure' to keep the blood under constant conditions against the action of the external media. Since, however, an increase of 74 per cent. in the osmotic pressure of the external fluid produces a much less increase in that of the blood, only 3·9 per cent. in the case of the cod, the amount of water passing from or into the fish must not be large. It is, however, large enough to show that the blood of the teleosts is not altogether independent of the external medium and a considerable alteration in the constitution of this latter is accompanied by a corresponding alteration in the constitution of the blood in defiance of any organs working to maintain this constitution constant.

Summary

1. The blood of marine teleosts has a considerably higher osmotic pressure than that of freshwater teleosts.

2. The change of density in the sea-water from Baltic to the North Sea is accompanied by a change, though small, in the osmotic pressure of the blood.

3. This same change occurs when a common eel is taken from fresh water and placed in sea-water, the osmotic pressure changes from that typical of freshwater teleosts to a much higher pressure, about the average for typical marine teleosts.

4. These changes indicate a permeability of the bounding membranes, probably only the gills, to water.

5. The teleosts contrive to maintain, partly by physiological

means, an osmotic pressure for the blood which is almost independent of the external water, and only great changes in the constitution of the surrounding medium affect this constancy.

6. Though considerable variations occur in any one species of teleost, in water of the same density, yet there is a 'mean' for the osmotic pressure of the blood which is distinct and peculiar to the respective species.

7. Different species react differently to the same changes in the outer medium.

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THE EFFECTS OF VARIATIONS IN THE INORGANIC SALTS AND THE REACTIVITY OF THE EXTERNAL MEDIUM UPON THE NUTRITION, GROWTH, AND CELL-DIVISION IN PLANTS AND ANIMALS

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The experiments of which the results are here recorded may be regarded as a continuation of the work of Moore, Roaf, and Whitley[1] on growth and cell division as modified by the presence of alkali or acid sufficient to cause only slight changes in the chemical reactivity of the cell.

The earlier experiments were carried out on the fertilized eggs of *Echinus esculentus*, and it was found that while small traces of acid repressed growth and stopped cell-division, small traces of alkali stimulated the division of the cell, and slightly increased amounts rendered the division of the cell and growth of the organism highly irregular and pathological.

In the present experiments attempts were made to carry on these artificial stimulations of the cell, in higher plants and animals, including mammalia, and although the results are not in all respects like those in the simpler and unprotected organism of the echinus, they are in our opinion sufficiently interesting to merit description.

In the earlier stages of the developing echinus, one has practically a naked mass of protoplasm, the cell membranes being very thin and delicate, and under such conditions the cells have little or no protection against the attack of the chemicals placed in the water in which it is suspended, so that the result obtained is the immediate reply of the

1. *Proc. Roy. Soc.*, B. Vol. LXXVII, p. 102, 1905.

living cell to the added reagents. In the higher animal, and probably in the highly developed plant, however, there is the secretory and excretory activity of the cells themselves to be reckoned with, and the mutual protection and assistance that they give to one another, whereby alkali is neutralised by synthesised acid and *vice versa*, and where excess of either alkali or acid is rapidly drained out of the circulation and out of the tissue fluids by the action of the excretory organs. Although, as will be shown later, by giving large doses of alkaline or acid salts, the reactivity of the blood may for the time be considerably changed, yet this occurs in a very mediate fashion, and not with the crude directness found when the alkali or acid are added to the fluid in which the cell is bathed. One circumstance which may be specially mentioned is that the fluid in which the cells of the higher organisms live is full of organic matter and especially of proteins, these possess the property of acting according to the demands of the situation either as acid or as alkali, and so effecting the corresponding neutralization of either alkali or acid. This powerful factor against the disturbance of the natural equilibrium has to be constantly borne in mind in such experiments as we have to deal with here. As a result of its operation, the concentration of either hydrogen or hydroxyl ions can never, fortunately for the living cell, be raised very high, and it is difficult to judge from changes in reactivity after incineration what the values were before incineration and in the presence of the proteins.[1]

Unfortunately, there exists no method by which the ionic concentrations, or even the reactivity to indicators, can be satisfactorily determined in the natural conditions of the medium bathing the cells.

We may infer from the experiments on echinus that the range of variation to either the acid or alkaline side compatible with life at all, is very short, and hence in the experiments recorded below the varia-

1. The question as to the maintenance of a constant reaction has been studied by L. J. Henderson, *Amer. Journ. of Physiology*, Vol. XXI, p. 427, 1908. This author has investigated the $\overset{+}{H}$ and $\overset{-}{Ho}$ concentrations in mixtures containing H_2CO_3, $NaHCO_3$, Na_2HPO_4, and NaH_2PO_4, and he shows that these are very efficient for maintaining a low concentration of $\overset{+}{H}$ and $\overset{-}{Ho}$. Other substances, such as the proteins, play a minor part in the reactivity regulation.

tions must have been well within this short range in spite of the large doses sometimes given.

There is at work a compensatory mechanism much like that which regulates the body temperature, and the effect of even our large doses of alkali or acid, is but to set the level a little higher or a little lower, as exposure to extreme variations of temperature will set the body temperature a degree or so up or down.

Even this slight change in the level of the gauge of the reactivity of the blood, if it may so be expressed, has, as will be shown, a profound effect upon the life and functions of the cells, so sensitive is the cell to the reaction of the medium in which it lives.

Just as in a warm-blooded animal a few degrees above the normal temperature causes the most violent disturbance in the metabolism, and disarranges the whole mechanism, so in the animal where the reactivity or ionic concentrations in the medium are nicely regulated even the small disturbance in the balance which can be brought about in spite of the regulating mechanism, leads to profound effects.

The experiments have been made on such widely different types of living cells that it will be convenient to describe them in separate sections.

A.—On the Effects of Acids and Alkalies and of Acid and Alkaline Salts on Growth and Cell-Division in Vegetable Cells

The plants used were a common variety of hyacinth (*Hyacinthus orientalis*) with pink florets, and the common onion (*Allium cepa*). Healthy bulbs of as nearly a uniform size as possible were chosen for the experiments. These bulbs were at first placed upon clean damp sand until the roots had commenced to grow, when they were transferred to hyacinth glasses which had been blackened on the outside with black lacquer to prevent action of light upon the rootlets. The hyacinth glasses held about 500 c.c., and the various solutions mentioned below in the desired concentrations were filled in until the solutions came just short of wetting the bulbs. The solutions were made up of fractions of gramme-molecular strength in Liverpool tap water[1] and

1. This is a very pure surface water, and practically free of inorganic salts.

were changed from time to time. A few of the ends of the growing roots were cut off at certain intervals for the purpose of studying the effects of the solutions on cell-division, and nuclear changes, and all these were immediately fixed with Flemming's strong solution; cut in paraffin; and stained for nuclear figures by Heidenhain's 'Iron-alum, Haematoxylin Method.' (See Plates.)

In addition to preparing the rootlets in this manner measurements and observations were made at intervals of the gross growth and condition of the plants, the points noted being the length of the green leaves, the length of the flower spike, the condition of the roots, the stage of development of the flower, and condition of the florets, whether all green, pink, open or closed, form of whole flower head, etc. These observations and measurements as well as the concentrations of the solutions used both in gramme-molecular strength and in percentages are given for the hyacinths in the accompanying Table (Table I). The results obtained in the case of the onions, where the work was chiefly confined to the preparing and examining of the root sections, are precisely similar to those with the hyacinth, and hence need no separate description.

The figures in the table printed in thick type show the stimulation of growth above the normal, which occurs chiefly with the alkaline solutions, potassium hydrate (No. 8), and alkaline sodium phosphate (Nos. 18 and 19); on the other hand the marked deleterious effects of even slight traces of acid is shown by the low figures marked by brackets in the case of Nos. 5 and 6.

Attention may be especially directed to several interesting points regarding rate of growth brought out by the table. First, the very small amount of free alkali or acid necessary to produce most marked effects is seen, and it is also shown that the range of variation in hydrogen and hydroxyl ions compatible with growth is a very narrow one. Secondly, much more of the carbonates and phosphates can be borne, but here the swing in hydrogen and hydroxyl ionic concentration is much less sharp, which is the probable explanation of this result.

There is nowhere seen a stimulating action with even the weakest

Table I.—Effects of Acid and Alkaline Substances on Growth of Hyacinths

(*a*) After seventy-one days' period of growth (November 14th, 1905—January 24th, 1906).

Substance and Concentration	Length in Centimetres: Green leaves	Flower spike	Remarks on condition of plant
1. Control	8·5	4·4	All green
2. Sodium hydrate (NaOH) 0·001 M = 0·004 %	8·2	4·6	Slight traces of pink
3. " " " 0·0015 M = 0·006 %	7·9	4·4	Very slight traces of pink
4. Hydrochloric Acid (HCl) 0·00075 M = 0·00274 % ...	7·6	5·0	Very slight trace of pink; some roots macerated
5. " " " 0·001 M = 0·00365 % ...	(2·8)	(2·0)	Very shortened green leaves and abortive spike; roots all macerated
6. " " " 0·0015 M = 0·00547 % ...	(2·0)	—	Practically no growth; no spike formed; root rotted off
7. Potassium hydrate (KOH) 0·001 M = 0·0056 % ...	7·1	3·7	All green
8. " " 0·0015 M = 0·0084 % ...	**10·3**	3·7	All green
9. Sodium bi-carbonate ($NaHCO_3$) 0·0015 M = 0·0126 %	7·7	4·1	All green
10. " " " 0·003 M = 0·0252 %	5·6	2·6	All green
11. " " " 0·005 M = 0·042 % ...	5·3	3·1	All green
12. " " " 0·01 M = 0·084 % ...	5·3	3·2	All green
13. Sodium carbonate (Na_2CO_3), 0·0015 M = 0·0159 % ...	7·0	4·4	All green
14. " " " 0·003 M = 0·0318 %	6·6	4·1	All green
15. Mono-sodium phosphate (NaH_2PO_4) (acid phosphate) 0·0005 M = 0·006 %	8·9	5·0	All green
16. " " " 0·001 M = 0·012 %...	7·9	3·8	All green
17. " " " 0·0015 M = 0·018 %	4·8	3·1	All green; roots slightly macerated
18. Di-sodium phosphate (Na_2HPO_4), (alkaline phosphate) 0·0025 M = 0·035 %	7·6	6·1	Great deal of pink colour in closed florets
19. " " " 0·005 M = 0·07 %	**11·1**	5·6	**Many pink florets, and some commencing to open**
20. " " " 0·01 M = 0·14 % ...	8·3	4·8	A few florets, very faintly pink

(*b*) The following observations were made on the 76th, 79th, and 86th days of growth, the growth and development of the flowers having occurred rapidly; the solutions and their concentrations being given above are not here repeated, and can be identified from the numbers.

	Seventy-six Days' Growth			Seventy-nine Days' Growth			Eighty-six Days' Growth		
	Length in cm. of		Remarks	Length in cm. of		Remarks	Length in cm. of		Remarks
	Leaves	Stalks		Leaves	Stalks		Leaves	Stalks	
1.	11·1	5·3	Florets turning pink	12·3	6·1	Lower florets open; intermediate ones green to pale yellow and fading	12·9	7·2	As before
2.	10·0	6·1	One or two florets nearly out	11·4	8·5	Almost all open	13·3	**11·3**	All open
3.	12·7	5·9	Five florets open	12·9	6·8	More florets open than control, but green on one side	13·8	7·2	One side still green, rest open; closely packed and irregular flower
4.	9·3	6·6	Five florets open	10·5	8·4	Nearly all open	11·4	**10·4**	All open
5.	(3·1)	(2·5)	Small, green, abortive	(3·1)	(2·6)	No development	(3·1)	(2·8)	No development
6.	(2·5)	—	Small, green, abortive	(2·5)	—	No development	(2·5)	—	No development
7.	11·1	4·6	All green	12·5	5·3	Slightly pink	13·6	5·8	A few open
8.	**14·2**	4·9	All green	**15·9**	5·8	Many pink, but none open	**17·7**	8·0	A fair number open
9.	9·5	4·9	A few florets tinged with pink	10·5	5·3	A few pink, none open	11·7	6·2	Lower open; top green
10.	8·8	4·1	All green	9·4	4·2	All green	10·9	4·5	All green, but well developed and healthy.
11.	7·2	4·6	All green	8·2	5·5	A few pink; three almost open	8·7	7·5	Nearly all open
12.	7·0	3·9	All green	8·3	4·4	All green	9·1	5·0	All green, but healthy
13.	10·4	5·0	A few showing pink	11·5	6·0	Three open, and a few slightly pink	13·1	7·2	A fair number open
14.	9·7	5·3	All green	10·4	6·7	Two open, some others pink	10·8	8·5	Nearly all out
15.	11·4	**7·9**	All pink, ten open	12·8	**11·0**	All open	**14·9**	**12·0**	As before
16.	10·7	5·4	All green	11·4	6·1	Several pink; none open	13·9	7·5	Lower open; top green
17.	7·5	2·8	All green	8·0	4·2	All green	8·1	4·9	All green
18.	10·3	**12·8**	**All florets open**	11·3	**17·2**	As before	**14·6**	**21·3**	All out and well expanded; best of series by far
19.	**15·0**	**9·0**	Nearly all open	**15·6**	**11·9**	All open	**17·2**	**14·6**	As before
20.	10·8	5·6	Lower florets open, top of flower yellowish and withered	13·0	6·3	Lower florets out, but flowers irregularly developed; florets packed together from non-development of the stalk, and unhealthy looking	14·0	6·6	As before

acid concentration used, and the histological examination (see next section) showed that even this minimal concentration in hydrogen ions damaged the root-tips and caused degeneration. On the other hand with the lower concentrations of alkali and alkaline phosphates (Nos. 18 and 19), there is distinct stimulation to increased rate of growth, which is confirmed by the histological investigation of the rootlets.

It is very noteworthy that the metallic ion seems to have a specific effect upon the rapidity of growth of the green leaves as distinct from the flower stalk. This is quite visible in the plants grown in the potassium hydrate solutions as No. 7, and more obviously still in No. 8.

The phosphatic ion appears to have a more special effect upon the flower stalk, causing a great increase in length at the optimum strengths, and a peculiar crowding together and irregular inflorescence due to packing together of the florets on a dwarfed stalk. This arrested development of the flower stalk, and very irregular and unequal development of florets was particularly well marked in No. 20, the strongest concentration of the alkaline phosphate used.

B.—Histological Investigation of the Growing Cells and Dividing Nuclei Under the Influence of the above Reagents

The accompanying micro-photographs show clearly the profound changes in the growing cells and in the rate and character of the nuclear divisions in the rootlets, accompanying the changes in the external medium. In general terms it may be stated that the smallest amount of acid used arrests the growth and nuclear divisions at the root tips ; causes the nuclei to become very obvious, but at the same time destroys their finer structure ; the cyptoplasm becomes hyaline, shrinks, and is destroyed, and the cell walls are clear and well marked. In addition to these effects the individual cells become enormously swollen, the linear dimensions being nearly doubled so that the cell volume is about eight times as great. The result is that the whole root tip becomes thicker. Thus all the micro-photographs in each set are of the same magnification, but the tip in B and J, the acid preparations, is enormously enlarged, and the higher magnifications

in the Sections B_1 and J_1 show that this is due to the enormous increase in the size of each cell.

The dilute alkali, on the other hand, stimulates to excessive nuclear division, the lines between the cells become almost or quite invisible, and the whole root tip comes to look like a syncytium with rapidly dividing nuclei. But examination further up the root shows that the cell outlines are visible and hence that the syncytium appearance is probably fallacious. The amount of chromatin under the influence of the increased hydroxyl ion concentration, becomes largely increased, the chromosomes becoming much thicker and more massive. There is also a great tendency to shortening of the chromosomes into rounded masses or dots, and the size and amount of chromatin in the different nuclei become exceedingly variable. It has been impossible for us to make out with any certainty the number of chromosomes in the dividing nuclei either in the normal rootlets or in those treated with the alkaline salts, but both the number and the shape of the chromosomes appear to vary much more widely in the rootlets treated with alkali than in the normal rootlets.

There appear to us to be two distinct forms of nuclei present in two different types of cell in both normal rootlets and chemically treated rootlets, one the normal parenchyma cell occurring in long rows parallel to the length of the root, and the other a cell with a longer and more prominent nucleus occurring intercalated between these rows, and appearing to possess a larger development of chromatin and a much greater number of chromosomes.

The following are brief notes of the histological examination of slides stained by Heidenhain's method of each of the Nos. 1 to 20, grown as above described. The roots were cut off and fixed, all at the same time, after seventy-two days' growth in the solution.

Control (No. 1).—The cells are arranged in regular rows with very clearly seen walls marking off their outlines. The number of actively-dividing cells is small. Most of the nuclei are in the resting condition, and in nearly all these resting nuclei there are seen two darkly staining dots resembling nucleoli each surrounded by a small clear space.

In addition to the ordinary parenchymatous cells, with round or oval nuclei which make up the greater part of the section, there are, especially in the median portion of the rootlet, cells visible with very elongated nuclei, but little cytoplasm, and no very obvious cell outline or cell wall. These cells are much more frequently in cell division compared to their numbers, and the chromosomes and division figures appear to be different, the amount of chromatin being greater than in the ordinary cells, and the chromosomes longer, more bent, and twisted upon themselves, and less regularly arranged.

In the subsequent sections treated with alkali the number of these cells is often enormously increased. In position these cells appear to be intercalated between the continuous rows of ordinary cells, to form discontinuous rows.

Sodium Hydrate (Nos. 2 and 3).—In No. 2 there is no very marked increase in the number of actively-dividing cells ; but where division is in progress the chromosomes appear to be thicker and shorter. These dividing nuclei are found chiefly in the second type of cell above mentioned, which has increased in number relatively to the other type, and many of the nuclei are exceedingly elongated. The nuclei of the resting cells are very prominent and longer than the normal, and the chromatin appears somewhat granular. In the mid region and at the tip the cell outlines are entirely invisible, and the nuclei appear to be closely studded together in a kind of syncytium.

The above differences are exaggerated in the stronger alkali of No. 3 (see micro-photographs B and B_1), the number of dividing cells being greater ; the chromosomes in some cases are shorter and thicker, in others they are long and thin, and curved upon themselves into loops and very irregularly disposed in the division. In the resting nuclei the black dots are very conspicuous, and the size of the surrounding clear space is increased ; occasionally the dot persists amongst the chromosomes in the dividing nucleus, but in the majority of cases it is missing.

Hydrochloric Acid (Nos. 4, 5, 6).—There is not a single dividing cell to be seen in any of the three strengths of acid, and most of the rootlets are degenerated. Thus even the most dilute acid, less than

0·003 per cent. (0·00075 M.), is an intense deterrent to cell vitality. In the few cases where degeneration is not absolute the cell walls are most clearly visible and thickened, the cytoplasm is granular, and the nuclei shrunken and rounded. The volume of the cell is also much increased (see micro-photographs C and C_1, which are exactly the same magnification as the others in each series).

Potassium Hydrate (Nos. 7 and 8).—In these sections the effects of alkalinity are more pronounced than in those from sodium hydrate as if the potassium ion had an additive effect (see micro-photographs D and E, and D_1 and E_1). The number of dividing cells is greater even in the more dilute strength than in the stronger solution of sodium hydrate. In the stronger potassium hydrate solution, there is seen a blurred mass of dividing nuclei in all stages of division on a common background of finely granular cytoplasm, showing no indication of discrete cells. The nests of chromosomes seem scattered about without any definite order; there are all grades of thickness and length of chromosome and spireme threads, and the contortions and shapes taken by the twisted chromosomes are very manifold. The black chromatin dots in the nuclei are of very variable sizes, and the surrounding clear space very conspicuous.

Sodium Carbonate and Bi-carbonate (Nos. 9 to 14). (See micro-photographs F, G, H and F_1, G_1, H_1).—The same effects are seen as in the case of the free alkalies, but occurring more gradually and only with much more concentrated solutions. The number of dividing nuclei is in all cases greater than in the normal, and in the bi-carbonates (Nos. 9 to 12 inclusive) a progressive increase in the number of dividing nuclei is seen *pari passu* with the increasing concentration of the sodium bi-carbonate. In the bi-carbonate series there is no appreciable effect upon the cytoplasm or cell boundaries, the walls are clearly marked, the rows of cells are beautifully regular, and there is no obvious increase in the number of the elongated nuclei of the median cells above referred to. The number of dividing nuclei of the ordinary parenchymatous cells is, however, very obviously increased above the number in the control, the increase throughout the series is also striking. In some members of the series the nucleolus-like dots are very

conspicuous, two or three in each cell surrounded by very clear spaces.

In the case of the normal carbonate, as might be perhaps expected from its more marked alkalinity, the effects are much more pronounced. The cytoplasm is much more granular, the regularity of arrangement of the cells in columns is considerably interfered with, the number of cells at some stage in the process of division is remarkably large. Even in the resting condition, the chromatin is very coarsely granular, and the chromatin threads in the fine skeins are very distinctly beaded in appearance. The chromosomes wherever present vary enormously in length and thickness, and in the shapes into which they are twisted and contorted. There is also no regularity in arrangement of the chromosomes into any dividing figure.

Mono-sodium-Phosphate or Acid-Phosphate (Nos. 15 to 17). (See micro-photographs I and J, and I_1 and J_1.)—In all three of these preparations the regularity of arrangement of the cells is beautiful, and although the two more dilute solutions show more nuclei dividing than the control, there is none of that disordered and incoordinated division of the alkaline preparations, the arrangement of the planes of division is perfect and regular, and there is no such variation in the chromosomes. The cell walls are more marked and clear than in the normal, and this culminates in the strongest solution in very much thickened walls which are brownish in colour in distinction to the blue-black staining of the chromatin of the nuclei. There is not a single dividing nucleus visible anywhere in the highest concentration, and the chromatin is very distinctly granular. There is a clear space, resembling the retraction space of the acid preparations, around each nucleus, and the cytoplasm is also granular, and has a precipitated appearance. A good many of the rootlets in this concentration are completely degenerated.

On the whole there is a close resemblance between the strongest acid phosphate and the acid preparations, while the weaker acid phosphates probably demonstrate the effects of the more minute increases in hydrogen ion concentration.

Di-sodium-Phosphate or Alkaline Phosphate (Nos. 18, 19 and 20).

(See micro-photographs K and L, and K_1 and L_1.)—The alkaline effects are not so characteristic as in the case of the free alkalies or the normal carbonate, as might perhaps be expected from the low degree of alkalinity (or hydroxyl ion concentration) of the solutions of this salt. Still, there is a noticeable increase in the frequency of dividing nuclei, but the divisions are well coordinated and the cells arranged in quite regular rows. There is no dimming or disappearance of the cell outlines which are as well marked as in the control. There are, however, more variations in length, thickness, and arrangement of the chromatin in the chromosomes and skeins than in the control, and an increase in the number of the median elongated nuclear cells, which are nearly all at some phase in division.

C.—The Effects of Phosphatic Solutions on the Rate of Growth in Amphibia (Japanese Newts, *Triton pyrrhogaster*)

The marked effects of phosphates upon growth in both vegetable and animal organisms which we had observed in the sea-urchin eggs[1], in the tadpole[2], and in the experiments recorded in Section A in the hyacinths made it desirable to test the action more thoroughly in more highly organised animals.

There are two different effects to be thought of chiefly in such experiments with acid and alkaline phosphates, the first being the variation in the reactivity of the medium, and the second the excess of the phosphatic ions, which is present both when acid and when alkaline phosphate are being used. The change in reactivity due to variations in hydrogen and hydroxyl ion concentrations, is largely met in the more highly organised animals by the altered metabolism of the animal, and hence whether acid or alkaline phosphate be employed, apart from the necessary effect upon metabolism, the chief factor of change in the inorganic constituents of the medium of the cell is the excess of phosphatic ions.

It is from this cause that the effects observed bear such a close

1. Moore, Roaf and Whitley, *loc. cit.*
2. Roaf and Whitley, *Bio-Chem. Jour.*, Vol. I, p. 88, 1906; and Roaf, *ibid.*, Vol. I, p. 383, 1906.

resemblance for the two series of phosphatic salt solutions, both in the experiments in this section, and in those in Section C on mammals, unlike the experiments on the echinus eggs and in lesser degree on the vegetable tissues where the most prominent change is the change in reaction, and the less prominent one the phosphatic excess.

For this series of experiments a gross of Japanese newts were purchased, and a dozen of each placed in each of a dozen of large flat circular specimen jars, such as are used for mounting anatomical or museum specimens. The jars gave ample room for air and water, being ten centimetres in depth and thirty centimetres in diameter. The glass lid of each dish was supported on small chips of wood so as to allow free access to air without allowing the newts to escape, and in each jar a piece of clean old brick was placed so that the newts could at will leave the solution and rest upon this brick, the top of which stood out of the solution.

At first the animals were kept in 400 c.c. of solution in each dish, which was just enough to cover the bottom, and the bodies of the newts, their heads being well out of the solutions. But later 1600 c.c. of solution was used.

The animals were fed on minced raw beef twice a week or occasionally on worms instead, and the solutions were always changed the same day after they were fed, to prevent putrefaction of unused food in the solutions. The animals lived in good health under these conditions except in one vessel, where there was an accident, probably due to poisoning by a small amount of meat decomposing in the vessel; as a result the population of this vessel was reduced to two newts. In all the others the mortality was low and there were ten or eleven newts left alive in each at the end of the experiment which went on for over ten months time.

The animals were weighed about once every fortnight, but in order to save space in the table only monthly weighings are here recorded. The weighing was done under exactly similar conditions in each case in the following manner. The superfluous moisture was drained off all the newts in any given solution and these were placed in a tared beaker and weighed. The total weight was divided by the

number of newts, so giving the average weight of the newt in that particular solution. Table II gives for each date the percentage gain in weight of the average newt calculated as a percentage on the original average weight of the newt at the commencement of the experiment. This table gives the best idea of the rate of growth in the various concentrations of solution, but for purposes of comparison the actual average newt weight in each solution is shown in Table III.

Regarding the conditions obtaining in this series of experiments it may be remarked that it is quite impossible to feed by hand such small animals with the phosphates. It is improbable that the skin served to any appreciable extent as a medium for uptake of the phosphates, but the food of the animals was placed in the solutions and there is no doubt that a good deal was taken up in this way, and that the amount of phosphate taken up would be proportional to the concentrations of the dissolved salts.

The strengths of solution taken were designedly low so as not to rapidly injure the newts, and to produce a slow chronic effect.

Two controls were used in the series of twelve dishes in which tap water only was used ; these were numbers 1 and 12 in the Tables ; No. 12 did not grow rapidly at first but caught up later, and the percentage increases at the end of 60·4 per cent. in No. 1, and of 62·0 per cent. in No. 12, are quite close together.

Acid phosphate of sodium (NaH_2PO_4) was added to the four lots of newts in Nos. 2, 3, 4 and 5, in concentrations of 0·0005, 0·0015, 0·0030 and 0·0050 gram-molecular concentration respectively ; these figures correspond to the percentages of 0·006, 0·018, 0·036, and 0·060 of the dried NaH_2PO_4.

Six lots of newts were grown in the alkaline phosphate of sodium (Na_2HPO_4) in concentrations of 0·00025, 0·00075, 0·00150, 0·00250, 0·00500, and 0·00750 gram-molecular concentration respectively, corresponding to percentages of the dry salt Na_2HPO_4 of 0·0035, 0·0106, 0·0213, 0·0355, 0·0710, and 0·1065 respectively.

These concentrations were chosen from the experience of our previous work as those compatible with cell life, and below the limits likely to cause rapid damage. When regard is paid to the small per-

centages added to the solutions, the effect on growth recorded in the Tables is remarkable.

Both the acid and the alkaline phosphates at a certain optimum concentration cause a well-marked increase in the rate of growth. The reason why both act alike in this respect has already been touched upon. The optimum for the acid phosphate lies at 0·0015 to 0·0030 M, and that for the alkaline phosphate at about 0·00075 to 0·00150 M.

Attention may further be drawn to the fact that the weights in all cases rose to a certain maximum and then declined; the maximum being reached for all early in August after which there is a general decline lasting to the end of the experiment on October 26th.

It is also highly interesting to note that the newts in the controls, and in solutions of concentrations both above and below the optimum, never did attain to the amount of increase in weight seen in the optimum, but merely increased at a certain slower rate until the August maximum and then went down with the others.

Effects of Higher Concentrations of the Phosphatic Solutions.—At the end of the experiments with the more dilute solutions which had been well borne throughout so many months, the same animals were used to test the more lethal effects of increased concentrations. Two sets of newts were taken, one in the acid phosphate and the other in the alkaline phosphate, and in each case the concentration was increased by definite amounts at regular intervals until lethal effects were produced.

Alkaline Phosphate.—The newts in 0·022 M. concentration were placed in the following successive concentrations at the intervals named :—0·030 M. for twenty-five days ; 0·037 M. for fourteen days ; 0·044 M. for three days; 0·050 M. for four days; 0·056 M. for five days. At the concentration of 0·056 M., the newts became strongly affected, and showed marked hyper-excitability passing into tonic contractions of a spasmodic character accompanied by excessive ophisthotonus when handled, and rapidly died off in this condition if left in the strong solution. If removed to water, they recovered. More exact experiments were instituted with more dilute solutions,

TABLE II.—PERCENTAGE GAIN IN WEIGHT OF JAPANESE NEWTS GROWN IN PHOSPHATIC SOLUTIONS

Solution used	Concentration of Solution: Gramme molecular	Per-centage	Date and Number of Days' Duration of Experiment (The figures give percentage gain in weight on original): Feb. 23, 43 days	Mar. 23, 71 days	Apl. 24, 103 days	May 25, 134 days	June 22, 162 days	July 20, 190 days	Aug. 3, 204 days	Aug. 17, 218 days	Sept. 14, 246 days	Oct. 26, 288 days
1. Control	Nil	Nil	20·75	41·48	51·44	68·54	80·24	79·81	**82·82**	74·55	98·89	60·40
2. Mono-sodium-phosphate (NaH_2PO_4, *acid* phosphate)	0·00050 M	0·006	24·98	48·18	35·55	66·75	75·95	79·68	**84·29**	82·49	83·99	67·59
3. ,, ,,	0·00150 M	0·018	**48·98**	**64·50**	**67·07**	**90·29**	**103·04**	**114·3**	**119·2**	**109·4**	**114·30**	**95·45**
4. ,, ,,	0·00300 M	0·036	26·62	48·59	56·58	74·87	85·04	104·38	**105·21**	98·45	108·19	95·19
5. ,, ,,	0·00500 M	0·060	20·92	37·79	45·85	56·88	63·45	79·90	**84·76**	78·59	78·60	66·84
6. Di-sodium-phosphate (Na_2HPO_4, *alkaline* phosphate)	0·00025 M	0·004	27·73	45·56	57·11	62·94	72·74	95·00	**82·69**	102·18	86·27	71·53
7. ,, ,,	0·00075 M	0·011	**34·59**	**52·42**	**56·92**	**66·74**	**88·03**	**89·67**	**91·64**	**102·33**	**89·17**	**82·25**
8. ,, ,,	0·00150 M	0·021	33·26	45·39	49·20	66·66	77·84	88·23	**88·66**	72·70	81·71	75·01
9. ,, ,,	0·00250 M	0·036	25·94	38·00	42·37	66·76	70·33	87·12	**91·18**	60·40	64·26	81·02
10. ,, ,,	0·00500 M	0·072	20·36	37·74	40·20	52·05	71·30	84·28	**84·51**	83·14	82·35	75·97
11. ,, ,,	0·00750 M	0·107	26·14	31·86	35·54	47·44	55·82	73·86	**75·52**	66·45	72·48	57·13
12. Control	Nil	Nil	1·13	24·35	50·25	51·50	62·35	72·70	**76·50**	70·48	70·80	62·02

The thick figures in the horizontal lines mark the optimum concentration of each salt; and the thick figures in the vertical line the percentage increases in weight, at the period of greatest weight (August 3).

TABLE III.—AVERAGE WEIGHT IN GRAMMES OF NEWT AT PERIODS SHEWN IN THE GIVEN SOLUTIONS

Solutions used Concentrations as in Table II	Original weights of Newts Jan. 11	Date and Period in Days from Commencement of Experiment Jan. 22 11 days	Feb. 23 43 days	Mar. 23 71 days	Apl. 24 103 days	May 25 134 days	June 22 162 days	July 20 190 days	Aug. 3 204 days	Sept. 14 246 days	Oct. 26 288 days
1. Control	3·725	4·121	4·498	5·370	5·641	6·278	6·714	6·698	6·810	7·408	5·975
2. Mono-sodium-phosphate (NaH_2PO_4)	3·991	3·969	4·988	5·914	5·410	6·655	7·022	7·171	7·355	7·343	6·688
3. " "	3·583	4·062	5·338	5·894	5·986	6·818	7·276	7·678	7·850	7·679	7·003
4. " "	3·542	3·753	4·485	5·263	5·546	6·194	6·554	7·239	7·273	7·374	6·914
5. " "	4·035	4·268	4·879	5·556	5·885	6·330	6·595	7·259	7·455	7·207	6·732
6. Di-sodium-phosphate (Na_2HPO_4)	3·859	3·857	4·929	5·617	6·063	6·288	6·666	7·125	7·050	7·188	6·619
7. " "	3·960	3·995	5·329	6·036	6·114	6·603	7·446	7·511	7·589	7·591	7·217
8. " "	3·882	4·086	5·173	5·644	5·792	6·470	6·904	7·307	7·322	7·054	6·794
9. " "	4·432	4·432	5·582	6·116	6·310	7·391	7·549	8·298	8·473	7·280	8·023
10. " "	4·390	4·898	5·284	6·047	6·155	6·675	7·520	8·090	8·100	8·005	7·725
11. " "	3·914	4·025	4·937	5·161	5·305	5·771	6·099	6·805	6·870	6·751	6·150
12. Control	4·000	4·143	4·045	4·974	6·010	6·042	6·494	6·908	7·060	6·832	6·481

and it was found that the lethal concentration for the alkaline phosphate (Na_2HPO_4) was 0·044 M.

Acid Phosphate.—Similar experiments were carried out here, the concentrations and times being as follows :—0·015 M. for twenty-five days ; 0·020 M. for fourteen days ; 0·025 M. for three days ; 0·031 M. for four days; 0·037 M. for three days; 0·044 M. for four days; 0·050 M. for seven days ; 0·056 M. for twelve days ; 0·063 M. for seven days ; 0·069 M. for thirty days. The animals in this solution never showed the hyper-excitability of the animals in the alkaline phosphate at any stage, but simply became more lethargic. This was most marked in the strongest solution 0·069 M., at about the end of a month in this solution they began to die off. At any period, if removed from the acid phosphate solution and placed in water they recovered their normal condition from this lethargy which looked very like an anaesthetic condition. Often, animals so inert as to appear dead, when placed in fresh water, were found next day moving and active.

The effects of the two phosphates are therefore different—resembling the effects on the cells—the alkaline salt giving before the lethal amount is reached, a hyper-excitation, while the acid salt is throughout depressant. The lethal doses are also different, being 0·044 M. for the alkaline salt and 0·069 M. for the acid salt. We were surprised at the conclusion of our experiments to find how closely these figures coincide with those found by Roaf and Whitley[1] in the case of the growing tadpole, being 0·046 M. for alkaline phosphate, and 0·075 M. for acid phosphate.

D.—The Effects of Administration of Alkaline and Acid Phosphates in Mammalia

The object of this series of experiments was the study of the effects of the acid and alkaline phosphates when administered in excess in mammalia, in view of the results obtained in lowlier animal organisations.

Although the primary purpose was to develop the full physio-

1. *Bio-Chemical Journal*, Vol. I, p. 97, 1906.

logical effect of the drugs, it was not at first adequately realised what a large percentage of water of crystallization is present in the crystalline salts, and hence, rather chronic effects were really obtained, these, however, are none the less interesting on account of the more slow development, and the histological changes in the tissues suggest a further series of experiments with smaller doses still so as to obtain even more chronic results.

In view of the fact that some forms of food, such as raw meats and bread, contain an excess in their ash of acid phosphates, such a slower series would be of high interest.

Whether the peculiar metabolic results of a diet restricted to meat alone, or to bread only, are due to the acid phosphatic ash or not, it is certainly a fact that excess of phosphatic ions either as alkaline or as acid salts exercises a profound influence on metabolism in general, and leads to marked chronic changes in the tissues.

In order to avoid any disturbing influence of the kation, the sodium salts were used in the experiments.

The animals employed were guinea-pigs as an example of a herbivorous animal, and dogs as a typical carnivorous animal.

Only one dog was used for each of the two salts (NaH_2PO_4, and Na_2HPO_4) since the experiments were somewhat prolonged; and twelve guinea-pigs in all were used for the other sets of experiments.

Experiments in Herbivorae (Guinea-Pigs)

Some initial difficulty was experienced in regard to the method of administration of the phosphates on account of the small size of the animals. The method which was finally found to work best, and which succeeded so well that some of the animals took the dose voluntarily, and the others without difficulty swallowed it when it was placed in the mouth, was as follows:—The drug is accurately weighed out, placed in a mortar and ground to a fine powder, sufficient water is added to make a thin paste, then ordinary wheat flour is added gradually and worked up to form a dough. The whole mass is divided into as many equal weighed portions as there are

animals to be treated, and then each portion of dough is shaped into small pellets, and given to the animal to eat, or placed one after another on the tongue, when they are readily swallowed.

The food, in addition to these dough pellets containing the phosphates, consisted of bran and water, and green vegetables.

The animals were given the phosphates, and fed, twice daily at 11 a.m. and 3 p.m., and were weighed daily at 12 noon.

Experiment I.—Each of five guinea-pigs received twice daily 0·4 gramme of the crystalline di-sodic-phosphate (Na_2HPO_4, $12H_2O$); this dose corresponds to 0·08 gramme of P_2O_5, or for the two doses to a daily administration of 0·16 gramme, or, taking 500 grammes as the average weight, to about 0·3 gramme per kilogramme of body weight.

The results of the experiment are shown in the following table (Table IV).

TABLE IV.—EFFECTS ON BODY WEIGHT OF ADMINISTRATION OF ALKALINE PHOSPHATE (Na_2HPO_4, 12 aq.)

Date	I 0·38 gram.*	II 0·35 gram.*	III 0·35 gram.*	IV 0·24 gram.*	V 0·30 gram.*
1st day	420 grams.	250 grams.	458 grams.	655 grams.	529 grams.
2nd „	392 „	455 „	456 „	650 „	517 „
3rd „	380 „	432 „	438 „	600 „	487 „
4th „	345 „	410 „	400 „	556	423 „
5th „		No phosphate given			
6th „	351 „	417 „	392 „	554 „	423 „
7th „	345 „	415 „	377 „	569 „	432 „
8th „	356 „	419 „	405 „	614 „	432 „
9th „	343 „	401 „	399·5 „	612 „	443 „
10th „	367·5 „	386·5 „	381 „	615 „	460 „
11th „	349 „	412 „	380 „	576 „	445 „
12th „		No phosphate given			
13th „	347 „	375 „	380 „	577 „	429 „
14th „	344·7 „	391 „	382 „	578 „	431 „
15th „	343 „	385 „	365·5 „	569 „	422 „
16th „	336 „	385 „	357 „	530 „	412 „
17th „	329 „	396 „	Death.	574·5 „	408 „
18th „	333 „	390 „	Decrease in body	620 „	393 „
19th „	327 „	390 „	weight 22 per	580 „	385 „
20th „	341 „	410 „	cent.	595 „	403 „
21st „	345 „	408 „	—	598 „	386 „

* Dosage in grammes per kilogramme of body weight in each case.

TABLE IV (*Continued*)

Date	I 0·38 gram.*	II 0·35 gram.*	III 0·35 gram.*	IV 0·24 gram.*	V 0·30 gram.*
22nd day	343 grams.	410 grams.	—	578 grams.	386 grams.
23rd ,,	343 ,,	400 ,,	—	607 ,,	377 ,,
24th ,,	343 ,,	416 ,,	—	613 ,,	379 ,,
25th ,,	351 ,,	430 ,,	—	561 ,,	392 ,,
26th ,,	354 ,,	425 ,,	—	570 ,,	378 ,,
27th ,,	345 ,,	428 ,,	—	597 ,,	336 ,,
28th ,,	353 ,,	421 ,,	—	638 ,,	Death.
29th ,,	339 ,,	419 ,,	—	582 ,,	Decrease in body
30th ,,	353 ,,	407 ,,	—	587 ,,	weight 36·5 per
31st ,,	379 ,,	435 ,,	—	604 ,,	cent.
32nd ,,	361 ,,	426 ,,	—	596 ,,	—
33rd ,,			No phosphate given		
34th ,,	362 ,,	426 ,,	—	622	—
35th ,,	368 ,,	428 ,,	—	626 ,,	—
36th ,,	348 ,,	414 ,,	—	613 ,,	—
37th ,,	334 ,,	374 ,,	—	513 ,,	—
38th ,,	354 ,,	390 ,,	—	558 ,,	—
39th ,,	356 ,,	348 ,,	—	545 ,,	—
40th ,,			No phosphate given		
41st ,,	355 ,,	358 ,,	—	570 ,,	—
42nd ,,	331 ,,	360 ,,	—	540 ,,	—
43rd ,,	350 ,,	372 ,,	—	565 ,,	—
44th ,,	344 ,,	386 ,,	—	587 ,,	—
45th ,,	346 ,,	382 ,,	—	573 ,,	—
46th ,,	361 ,,	385 ,,	—	571 ,,	—
47th ,,	301 ,,		No phosphate given		
48th ,,	Death.	317 ,,	—	558 ,,	—
49th ,,	Decrease in body	Death.	—	588 ,,	—
50th ,,	weight 28 per	Decrease in body	—	577 ,,	—
51st ,,	cent.	weight 29·5 per	—	550 ,,	—
52nd ,,	—	cent.	—	568 ,,	—
53rd ,,	—	—	—	547 ,,	—
54th ,,	—	—	—	460 ,,	—
				Death. Loss of body weight 29·8 per cent.	

The decrease in body weight in each case is very considerable, amounting on an average to a loss of 29·16 per cent. The other symptoms and results of post-mortem examinations and histological changes are given later.

* Dosage in grammes per kilogramme of body weight in each case.

Experiment II.—The next table illustrates the changes in weight of four guinea-pigs which were fed with the crystalline salt of the *acid* phosphate (NaH_2PO_4, 8 H_2O).

The amount of this salt given to each animal was 0·4 gramme twice daily.

This dose corresponds to 0·11 gramme of P_2O_5 or to 0·22 gramme for the daily amount administered, which for the approximate average weight of guinea-pig of 850 grammes used in the experiment corresponds to 0·26 gramme per kilogramme of body weight.

TABLE V.—EFFECTS ON BODY WEIGHT OF ADMINISTRATION OF ACID PHOSPHATE (NaH_2PO_4, 8 aq.)

Date	VI 0·24 gram.*	VII 0·26 gram.*	VIII 0·26 gram.*	IX. 0·29 gram.*
1st day	927 grams.	846 grams.	846 grams.	757 grams.
2nd "	891 "	841 "	830 "	771 "
3rd "		No phosphate given		
4th "	878 "	833 "	818 "	760 "
5th "	852 "	776 "	778 "	708 "
6th "	860 "	786 "	771 "	708 "
7th "	820 "	739 "	724 "	676 "
8th "	800 "	760 "	745 "	661 "
9th "	807 "	708 "	709 "	655 "
10th "		No phosphate given		
11th "	835 "	672 "	725 "	664 "
12th "	807 "	645 "	683 "	656 "
13th "	806 "	629 "	664 "	634 "
14th "	796 "	633 "	682 "	620 "
15th "	793 "	615 "	667 "	605 "
16th "	784 "	573 "	679 "	598 "
17th "	No phosphate given	Death. Decrease in body	No phosphate given	603 "
18th "	783 "	weight 32·3 per	692 "	593 "
19th "	806 "	cent.	709 "	510 "
20th "	780 "	—	681 "	Death.
21st "	787 "	—	700 "	Decrease in
22nd "	791 "	—	670 "	body weight
23rd "	772 "	—	695 "	32·7 per cent.
24th "		No phosphate given		
25th "	770 "	—	685 "	—
26th "	773 "	—	630 "	—
27th "	770 "	—	619 "	—

* Daily dosage in grammes per kilogramme of body weight.

TABLE V (*Continued*)

Date	VI 0·24 gram.*	VII 0·26 gram.*	VIII 0·26 gram.*	IX 0·29 gram.*
28th day	750 grams.	—	600 grams.	—
29th ,,	761 ,,	—	596 ,,	—
30th ,,	734 ,,	—	561 ,,	—
31st ,,		No phosphate given		
32nd ,,	753 ,,	—	597 ,,	—
33rd ,,	745 ,,	—	622 ,,	—
34th ,,	747 ,,	—	645 ,,	—
35th ,,	715 ,,	—	608 ,,	—
36th ,,	738 ,,	—	625 ,,	—
37th ,,		No phosphate given		
38th ,,	744 ,,	—	626 ,,	—
39th ,,	728 ,,	—	615 ,,	—
40th ,,	745 ,,	—	600 ,,	—
	Death. Decrease in body weight, 19·6 per cent.		Death. Decrease in body weight, 29·1 per cent.	

NOTE.—The table gives the decrease in weight under acid phosphate administration, the average loss in weight being 28·4 per cent.; other details are given later in the text.

Experiment III.—During the time that the animals in the above experiment were being treated with the acid phosphate, two more guinea-pigs were put on the alkaline phosphate; in this instance the animals were given double the quantity, *i.e.*, 0·8 gramme of Na_2HPO_4, 12 aq., equivalent to 0·16 grammes P_2O_5 twice daily.

One guinea-pig was kept as a control and not given anything except its ordinary food.

The results are shown in Table VI.

TABLE VI.—EFFECTS ON BODY WEIGHT OF ADMINISTRATION OF ALKALINE PHOSPHATE (Na_2HPO_4, 12 aq.)

Date	X 0·37 gram.*	XI 0·39 gram.*	XII Control
1st day	855 grams.	821 grams.	802 grams.
2nd ,,	818 ,,	855 ,,	827 ,,
3rd ,,		No phosphate given	
4th ,,	838 ,,	802 ,,	842 ,,
5th ,,	752 ,,	745 ,,	835 ,,

* Daily dosage in grammes per kilogramme of body weight.

TABLE VI (*Continued*)

Date	X 0·37 gram.*	XI 0·39 gram.*	XII Control
6th day	Death.	722 grams.	755 grams.
7th ,,	Decrease in body	702 ,,	804 ,,
8th ,,	weight 12·1 per	659 ,,	817 ,,
9th ,,	cent.	649 ,,	821 ,,
10th ,,	No phosphate given		
11th ,,	—	700 ,,	794 ,,
12th ,,	—	737 ,,	794 ,,
13th ,,	—	754 ,,	812 ,,
14th ,,	—	770 ,,	805 ,,
15th ,,	—	722 ,,	803 ,,
16th ,,	—	706 ,,	808 ,,
17th ,,	No phosphate given		
18th ,,	—	677 ,,	800 ,,
19th ,,	—	722 ,,	813 ,,
20th ,,	—	706 ,,	790 ,,
21st ,,	—	682 ,,	793 ,,
22nd ,,	—	685 ,,	787 ,,
23rd ,,	—	699 ,,	800 ,,
24th ,,	No phosphate given		
25th ,,	—	673 ,,	778 ,,
26th ,,	—	731 ,,	809 ,,
27th ,,	—	728 ,,	789 ,,
28th ,,	—	685 ,,	792 ,,
29th ,,	—	705 ,,	807 ,,
30th ,,	—	634 ,,	790 ,,
31st ,,	No phosphate given		
32nd ,,	—	652 ,,	760 ,,
33rd ,,	—	705 ,,	765 ,,
34th ,,	—	669 ,,	783 ,,
35th ,,	—	661 ,,	777 ,,
36th ,,	—	685 ,,	790 ,,
37th ,,	No phosphate given		
38th ,,	—	690 ,,	800 ,,
39th ,,	—	645 ,,	800 ,,
40th ,,	—	645 ,,	804 ,,
		Death. Decrease in body weight 21·5 per cent.	Killed, and tissues fixed and sectioned for control

SYMPTOMS OBSERVED DURING THE PROGRESS OF THE ABOVE EXPERIMENTS

As is shown by the tables given above the animals in all cases decreased in weight, the decrease being most rapid in the beginning and then more slowly with occasional variations in the upward direction, but on the whole showing a downward tendency until

* Daily dosage in grammes per kilogramme of body weight.

death ensued. The emaciation involved all the adipose tissue and also very extensively the skeletal muscles, the limb-muscles becoming excessively shrunken. Accompanying the decrease in weight a gradual enfeeblement of the animals occurred, so that they became torpid and remained almost motionless. It is interesting to note that although rapid decrease in weight occurred in the beginning of the experiment the animals were consuming more than the usual quantity of food, the appetite being so much increased that more than double the usual amount of food was taken. At a later stage the excretion of water by the bowel became increased so that the faeces lost their usual hard consistency and became very soft. It was noticed soon after the appearance of this soft condition of the faeces the animals lost their increased appetite and from this onward the amount of food taken decreased till it fell below normal, but the animals never reached a stage at which food was entirely refused.

Reactivity of the Blood.—In order to determine whether the drugs administered had altered the reactivity of the blood, a series of experiments was carried out by the method described by Moore and Wilson.[1]

Determinations were made in the normal animal, in animals under alkaline phosphate, and animals under acid phosphate.

TABLE VII

Guinea-pig	Drug administered	Alkalinity of blood
Number 9 ...	Control ...	$\frac{N}{30}$ H_2SO_4 = Normal alkalinity
Number 2 ...	Na_2HPO_4 ...	$\frac{N}{25}$ H_2SO_4 = Increased ,,
Number 10 ...	Na_2HPO_4 ...	$\frac{N}{25}$ H_2SO_4 = Increased ,,
Number 7 ...	NaH_2PO_4 ...	$\frac{N}{33}$ H_2SO_4 = Decreased ,,
Number 11 ...	NaH_2PO_4 ...	$\frac{N}{33}$ H_2SO_4 = Decreased ,,
Number 12 ...	NaH_2PO_4 ...	$\frac{N}{33 \cdot 5}$ H_2SO_4 = Decreased ,,

1. *Bio-Chemical Journal*, Vol. I, p. 297, 1906.

From the above results it will be observed that there was a marked increase in the alkaline reactivity of the blood in the animals receiving the alkaline phosphate, whilst on the other hand there was a decrease in the animals receiving the acid phosphate. The animals receiving the alkaline phosphate were not manufacturing sufficient acid to neutralise the alkali, and *vice versa* those receiving the acid phosphate were not manufacturing sufficient alkali to overcome the excess of acid.

It is hence obvious that the amounts of drug administered had been sufficient to alter the reactivity of the plasma in the direction intended in each series of experiments.

Post Mortem Examinations.—Post mortem examinations were made in twelve cases, in each case practically no adipose tissue was found subcutaneously or in the mesentery, the emaciation of the skeletal muscles was extreme, and the muscular substance pale in colour. On opening the abdomen, fluid was found in quantity in excess of normal in the peritoneal cavity. In some cases several cubic centimetres of clear amber coloured fluid was obtained. The fluid quickly coagulated spontaneously on removal from the body. It showed an alkaline reaction to litmus and 'di-methyl,' and acid to phenol-phthalein.

In three cases small ulcers were found in the stomach, three in number in two cases and two in the third case ; the ulcers were usually situated near the lesser curvature, were oval in shape and one to one and a half centimetres in diameter. Histological examination showed a close resemblance to acute gastric ulcer, the base was smooth, the edges clean cut, and the penetration extended to the submucosa. The liver was soft and friable, in some cases mottled and showing small yellowish patches on the surface. On cutting into the organ, small pale patches were noticeable in places. Portions of the organ were fixed (*a*) in Muller's fluid and formol in equal parts, and (*b*) in Flemming's fluid for histological investigation. The sections when cut and stained showed extensive and interesting changes which will be described in a separate section.

Extracts of the liver were made and tested for glycogen and

sugar, which were found to be abnormally low in quantity, the usual qualitative tests giving negative or doubtful results. The gall bladder was much distended in every case. The kidneys were usually pale in colour and in two cases showed small cysts on being opened.

Histological Examination of the Tissues

The Liver (*Alkaline Phosphate*).—The sections of the guinea-pig livers show in all cases degeneration of the liver cells, but the amount of degeneration varies in different animals and in sections from different parts of the same liver. In general the cells are vacuolated, and masses of round cells and polymorphonuclear leucocytes are observable throughout the tissue but especially well developed in masses lying around the vessels and about the portal canals, the vacuolation in certain places has progressed to the formation of larger cavities occupying nearly the whole of the cytoplasm. Complete destruction of cells is observable in places, and in certain situations rounded cavities larger than the liver cells are seen. In some sections well marked patches of tissue in process of necrosis are plainly marked off from more nearly normal tissue ; these patches vary much in size and in the extent to which necrosis has advanced ; they are usually marked off at their periphery by a thick layer of young connective tissue thickly studded with round cells and polymorphonuclear leucocytes. The tissue within the patches at times contains liver cells staining normally but showing vacuoles and cavities of different sizes ; in other cases the cells stain badly, taking on only a diffuse pink staining with eosin and showing no nuclei. In certain cases, on the outer margin are seen clusters of small round masses much larger than a liver cell which stain a deep blue with the haematoxylin. These are probably derived from nuclear chromatolysis of the liver cells, for similar dots are to be observed deeper in the necrosing islet spread out more uniformly in liver cells which are not so far advanced in degeneration. In some cases the growth of young connective tissue is enormously increased so that it occupies nearly the whole of the tissue. Between the strands of the growing connective tissue, large clear spaces are observable, and interspersed with large cells

occurring singly or in small groups which have the staining and appearance of liver cells; many of these cells are, however, larger than the normal liver cells, and in many cases they are multi-nucleated.

The Liver (Acid Phosphates).—The liver is enormously congested, and the cells are vacuolated. The vacuolation of the cells is, however, different from that seen in the case of the alkaline phosphates, the nucleus often being surrounded by a clear space or the entire cytoplasm of the cell takes on a very light staining. A considerable development of young connective tissue is seen around the portal canals.

The Kidney.—In sections of the kidney with both alkaline and acid phosphates there is acute nephritis particularly well marked in and around the glomeruli.

Gastric Ulcers.—The ulcers resemble an acute gastric ulcer with erosion of epithelium and formation of granulation tissue.

Experiments on Carnivora (Dogs)

Administration of the phosphates accompanied by determination of the metabolism of various products was undertaken in two prolonged experiments on dogs. One animal was given alkaline phosphate, and the other acid phosphate (See Tables IX and X).

The animals were kept in boxes of the usual form for experiments in metabolism. The animals were offered a diet of five hundred grammes of horse flesh daily, and were given as much water as desired; the total amount of meat was not always eaten; the daily amount of phosphate given is recorded in the tables. The urine was carefully collected, measured and analysed daily for chlorides, phosphates, total acidity, and total nitrogen.

The chlorides were determined by titration with standard solution of silver nitrate; the phosphates by the uranium acetate method; the total acidity by the phenol-phthalëin, and the total nitrogen by Kjeldahl's.

The method of feeding was the same as that employed in the case of the guinea-pigs.

The animals were weighed twice weekly.

TABLE IX.—EFFECTS OF ALKALINE PHOSPHATES ON METABOLISM

Date	Weight in grams.	Doses in grams. of as crystalline salt (Na_2HPO_4, $12H_2O$)	Dose in grams. as P_2O_5	Dosage of P_2O_5 per kilogram of body weight	Quantity of urine in c.c.	Chlorides expressed as grams. of NaCl	Phosphates P_2O_5	Acidity c.c. of deci-normal solution	Nitrogen in grams.
1st day	7650	none given	—	—	—	—	—	—	—
2nd "	—	" "	—	—	260	0·4570	1·3590	364	15·039
3rd "	7510	" "	—	—	275	0·4165	1·3254	350	13·126
4th "	—	" "	—	—	300	0·3867	1·5940	342	15·649
5th "	—	" "	—	—	250	0·4981	0·9396	205	16·235
6th "	—	2 grams.	0·4 grams.	0·053 grams.	350	0·4562	1·3527	280	13·691
7th "	7960	2 "	0·4 "	0·050 "	300	0·4219	1·2960	300	15·760
8th "	—	2 "	0·4 "	0·050 "	250	0·5860	1·2960	310	13·181
9th "	—	2 "	0·4 "	0·050 "	200	0·3819	0·9417	200	9·105
10th "	7800	2 "	0·4 "	0·051 "	250	0·6739	1·4364	255	13·587
11th "	—	3 "	0·6 "	0·077 "	300	0·6328	1·3089	300	17·748
12th "	—	3 "	0·6 "	0·077 "	160	0·3562	0·6912	285	11·533
13th "	7650	3 "	0·6 "	0·078 "	400	0·5625	1·3265	265	17·529
14th "	—	4 "	0·8 "	0·10 "	180	0·8432	0·8553	223·5	13·569
15th "	—	6 "	1·2 "	0·15 "	250	0·5026	1·5120	340	16·681
16th "	—	6 "	1·2 "	0·15 "	300	0·4219	1·9569	414	19·597
17th "	8050	8 "	1·6 "	0·2 "	400	0·6362	2·3096	420	23·844
18th "	—	none given	—	—	—	—	—	—	—
19th "	—	" "	—	—	—	—	—	—	—
20th "	—	6 grams.	1·2 "	0·15 "	300	1·2657	1·6329	328	15·314
21st "	7950	6 "	1·2 "	0·15 "	450	1·1057	2·0412	342	30·290
22nd "	—	8 "	1·6 "	0·2 "	500	0·6446	2·0520	380	19·712
23rd "	—	8 "	1·6 "	0·2 "	500	0·4688	2·6696	410	25·284
24th "	8450	10 "	2·0 "	0·23 "	400	0·5625	3·2000	488	21·078
25th "	—	14 "	2·8 "	0·33 "	450	0·8888	3·9506	458	21·987
26th "	—	14 "	2·8 "	0·33 "	550	0·6446	0·6446	460	26·996
27th "	—	20 "	4·0 "	0·47 "	560	1·3129	2·9030	442	35·082
28th "	8270	10 "	2·0 "	0·28 "	500	0·5860	4·9248	470	25·858
29th "	—	none given	—	—	—	—	—	—	—
30th "	—	10 grams.	2·0 "	0·28 "	350	0·3281	1·8900	480	18·600
31st "	8044	10 "	2·0 "	0·25 "	400	0·4219	3·0568	272	17·740
32nd "	—	10 "	2·0 "	0·25 "	300	0·5625	2·8081	330	14·364
33rd "	—	10 "	2·0 "	0·25 "	380	0·7125	2·1666	273·6	14·970
34th "	—	10 "	2·0 "	0·25 "	305	0·7506	2·6387	274·5	14·526
35th "	8050	10 "	2·0 "	0·249 "	390	0·8227	2·7753	280	16·838
36th "	—	10 "	2·0 "	0·249 "	485	0·7488	3·7452	403·2	22·610
37th "	—	10 "	2·0 "	0·249 "	290	0·5098	2·7568	220	13·162
38th "	—	10 "	2·0 "	0·249 "	640	1·2001	4·2866	499	28·152
39th "	8100	10 "	2·0 "	0·24 "	515	0·7102	3·9673	404	20·130
40th "	—	10 "	2·0 "	0·24 "	600	0·2812	3·5803	387	27·560
41st "	—	10 "	2·0 "	0·24	620	0·8719	3·2062	356	23·054
42nd "	—	10 "	2·0 "	0·24 "	510	0·7770	2·7726	397	16·936
43rd "	8070	15 "	3·0 "	0·37 "	—	—	—	—	—
44th "	—	20 "	4·0 "	0·5 "	450	0·6328	4·0973	380	19·000
45th "	8020	20 "	4·0 "	0·5 "	560	0·8536	4·771	448	24·633

Table X.—Effect of Acid Phosphates on Metabolism

Date	Weight in grams.	Doses in grams. of as crystalline salt (Na_2HPO_4, $12H_2O$)	Dose in grams. as P_2O_5	Dosage of P_2O_5 per kilogram of body weight	Quantity of urine in c.c.	Chlorides expressed as grams. of NaCl	Phosphates P_2O_5	Acidity c.c. of deci-normal solution	Nitrogen in grams.
1st day	9250	None given	—	—	310	3·2874	0·7094	—	5·9765
2nd "	—	" "	—	—	450	1·5030	1·2052	—	8·0996
3rd "	—	2 grams.	0·55 grams.	0·06 grams.	275	2·2318	0·6806	—	5·6743
4th "	—	2 "	0·55 "	0·06 "	320	1·3568	0·9450	—	7·9386
5th "	8520	2 "	0·55 "	0·064 "	300	0·7790	1·0303	—	8·4525
6th "	—	none given	—	—	—	—	—	—	—
7th "	—	" "	—	—	—	—	—	—	—
8th "	8290	2 grams.	0·55 "	0·068 "	325	0·9522	1·2144	—	10·6561
9th "	—	2 "	0·55 "	0·068 "	190	0·6235	1·0998	—	6·6504
10th "	—	2 "	0·55 "	0·068 "	275	0·7635	1·7760	—	8·9320
11th "	—	2 "	0·55 "	0·068 "	400	1·3360	1·3996	—	11·4688
12th "	7520	2 "	0·55 "	0·073 "	600	0·7383	1·2960	276	16·4304
13th "	—	2 "	0·55 "	0·073 "	500	0·3223	1·9224	320	7·0560
14th "	—	2 "	0·55 "	0·073 "	520	0·3656	2·4787	546	16·0014
15th "	—	2 "	0·55 "	0·073 "	360	0·4430	2·3405	460	12·2464
16th "	8040	2 "	0·55 "	0·068 "	400	0·2812	2·2464	508	17·3376
17th "	—	2 "	0·55 "	0·068 "	560	0·5240	2·1772	481·6	23·6454
18th "	—	none given	—	—	400	0·5854	2·0316	462	19·6588
19th "		2 grams.	0·55 "	0·068 "	320	0·3750	1·3962	476	12·6588
20th "	8180	2 "	0·55 "	0·067 "	500	0·6446	2·1816	504	18·6201
21st "	—	2 "	0·55 "	0·067 "	300	0·7383	1·6588	360	14·6587
22nd "	—	3 "	0·82 "	0·1 "	470	0·3385	2·5583	545·2	19·9246
23rd "	8570	3 "	0·82 "	1·05 "	300	0·4570	1·3089	258	12·7425
24th "	—	3 "	0·82 "	1·05 "	460	0·5930	1·7884	450	19·2942
25th "	—	4 "	1·1 "	0·13 "	400	0·5156	1·5206	520	20·1936
26th "	—	4 "	1·1 "	0·13 "	350	0·8204	1·2852	640	16·9834
27th "	8540	4 "	1·1 "	0·13 "	400	0·3281	3·2541	576	18·1664
28th "	—	6 "	1·6 "	0·19 "	550	0·5412	2·7324	605	24·8556
29th "	—	6 "	1·6 "	0·19 "	650	0·9753	2·6484	550	25·6432
30th "	8600	8 "	2·2 "	0·26 "	800	1·4064	2·8339	448	28·5152
31st "	—	—	—	—	—	—	—	—	—
32nd "	—	—	—	—	—	—	—	—	—
33rd "	—	6 "	1·6 "	0·19 "	650	1·1427	1·9656	450	35·1664
34th "	8390	6 "	1·6 "	0·20 "	600	0·5844	1·8921	480	24·9984
35th "	—	8 "	2·2 "	0·26 "	800	0·9376	3·1104	460	27·0592
36th "	—	8 "	2·2 "	0·26 "	—	—	—	—	—
37th "	8770	10 "	2·7 "	0·30 "	900	0·4219	1·5912	360	18·7056
38th "	—	None given	—	—	—	—	—	—	—
39th "	—	" "	—	—	1000	0·4688	1·9448	440	21·1120
40th "	—	14 grams.	3·7 "	0·42 "	900	0·8438	1·5912	542	13·0824
41st "	8220	10 "	2·7 "	0·33 "	850	0·7534	2·2564	550	9·5754

Attention may be drawn (*a*) to the polyuria which accompanies the administration of the phosphate, especially marked as the dose is increased; (*b*) to the increase of phosphates in the urine showing that the drug was being absorbed into the system. The amount of excreted phosphates in the urine always exceeds the amount given as a drug, the excess representing the greater part ingested with the food, showing that by far the greater part of the additional phosphate administered as a drug has been absorbed. There is no considerable change in the reactivity of the urine to phenol-phthalëin. The amount of total nitrogen excreted in the urine is considerably increased, especially with the high doses of phosphate. Throughout the greater part of the experiment it will be observed that the dosage of phosphate per kilogramme of body weight is considerably less than in the case of the guinea-pig experiments.

Although the animal's weight had not appreciably fallen during the interval, it was demonstrated by histological examination that important changes were commencing in the tissues, especially in the liver and kidneys, of which a detailed account is given below in the histological section.

Similar effects to those already noted in the case of the guinea-pigs of initial increase of appetite followed by diarrhoea and diminution of appetite at the later period, when the dose was increased, were observed in this experiment. The amount of water taken by the animal increased as the polyuria set in.

Diarrhoea made its appearance on the third day, and was modified later on although the faeces still remained soft. On increasing the dose the diarrhoea became exaggerated, and the animal did not eat so much food or vomited a portion after the meal.

Post-mortem Examination.—The chief macroscopic abnormal feature observed was ulceration of the duodenum and jejunum, a number of small clean-cut ulcers (twelve) were found, with a fairly smooth base penetrating to the submucosa. The ulcers were hardened and sectioned, and portions of liver and ulcers were hardened for histological examination.

Histological Examination of Tissues from Dogs

The Liver.—Degeneration has not proceeded so far in the livers of the dogs as in the case of the guinea-pig, probably because the dose per kilogramme of body-weight throughout the greater part of the experiment was not so high, and the animals were killed at an earlier stage. There is, however, a good deal of vacuolation in the cells; considerable amount of leucocytosis, which is more pronounced in the case of the alkaline phosphates, and the spaces between the liver cells are much greater than in normal liver tissue of the dog. The amount of connective tissue is also increased although not so excessively as in the guinea-pig livers.

The Kidneys in both cases show well-developed interstitial nephritis between the kidney tubules. In the dog on acid phosphate a large amount of round-celled tissue is also observable surrounding many of the glomeruli. Considerable numbers of polymorphonuclear leucocytes are present in the interstitial patches in both animals; but in the case of the dog on alkaline phosphates the majority of the leucocytes in the patches are polymorphonuclear. These leucocytes appear to be in a very active condition, and in the case of the dog fed on alkaline phosphate it is interesting to observe that in the interstitial patches there is a large number of cells of a different type. These cells are large, with lightly stained cytoplasm and oval-shaped nuclei. In the neighbourhood of the tubules they become mixed up with the epithelium of the tubules, the basal membrane surrounding the tubules having in places disappeared, and leucocytes are to be seen in between the tubule cells.

Intestine and Intestinal Ulcers in Dogs.—The sections show an erosion and removal of the villi and a clearly-marked area of round-celled infiltration and inflammatory tissue filled with small round cells.

Conclusions

Vegetable Tissues

1. Marked effects are produced upon the dividing cells of plant rootlets by small variations in the alkalinity or acidity of the medium. The range of ionic concentrations compatible with life and growth is a very narrow one.

2. Short of the lethal dose, there is a marked stimulation by the alkali which is not found with the acid.

3. The kation present appears to have a specific effect, and potassium is much more stimulating than sodium to both rootlets and foliage leaves.

4. The phosphatic anion has a special effect upon the flower, causing increase in size at optimum strength and at higher concentrations, an irregular inflorescence, with packed florets on a dwarfed stalk.

5. The cytological effects are best seen in the accompanying illustrations. There is absence or depression of nuclear division with the acid, and the thickening of cell walls; with the alkalies, increase in nuclear division, changes in chromosomes, and irregular figures are visible, and the cell outlines become obscured.

Amphibia

6. Both acid and alkaline phosphates at optimum concentrations cause increased growth in amphibia. Higher concentrations cause death; in the case of the alkaline salt only, preceded by a stage of hyper-excitability.

Mammalia

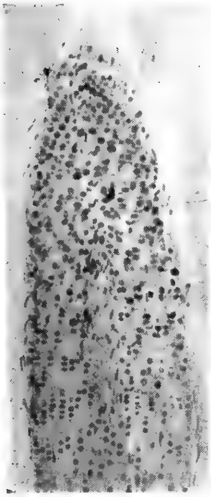
7. Either alkaline or acid phosphate in doses of 0·26 to 0·38 gramme per kilogramme of body weight causes increased metabolism with diminishing body weight, and finally death. At death, all fat has practically disappeared and there is prolonged muscular wasting.

8. There is polyuria; and with large doses, diarrhoea.

9. The alkaline reactivity of the serum is increased by the alkaline salt, and diminished by the acid salt.

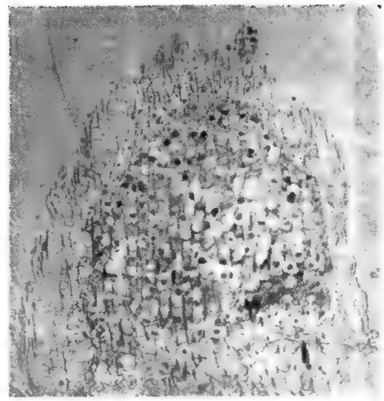
10. There is an irritative action upon the epithelium of the alimentary canal, leading often to ulceration.

11. Histological examination of the tissues shows in all the tissues excessive appearance of small round cells, especially in the neighbourhood of the blood vessels. In the liver there is profound degeneration of the liver cells, excess of embryonic connective tissue, and leucocytosis. In the kidneys there are patches of acute and of interstitial nephritis, and there are polymorphonuclear cells in abundance in the inflammatory patches.

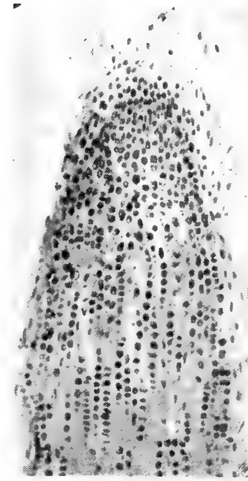


Section A

Section B

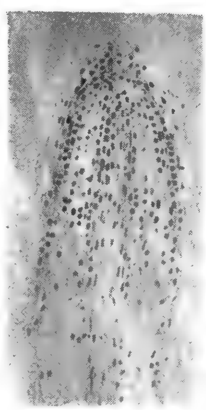


Section C

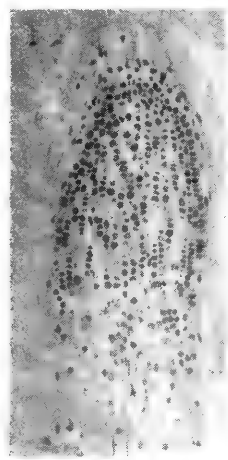


Section D

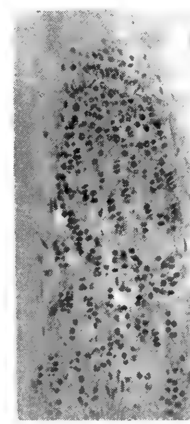
Section E



Section F

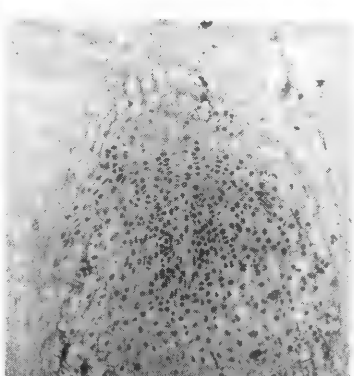


Section G

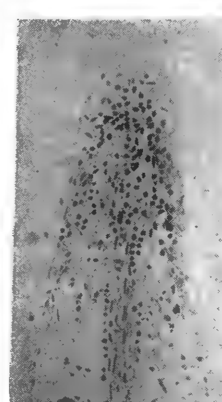


Section H

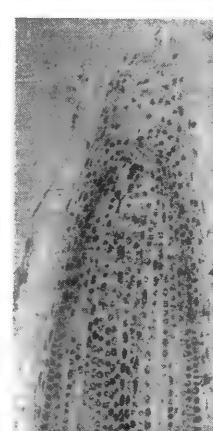
Section I



Section J

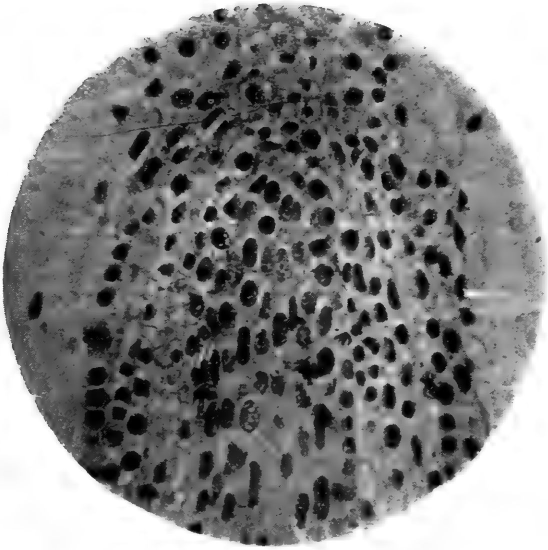


Section K



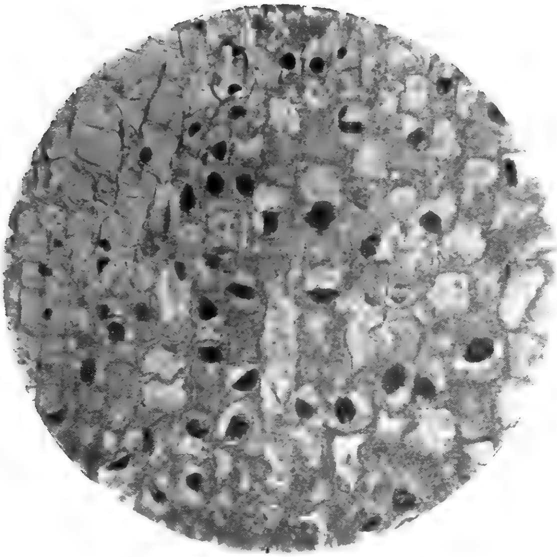
Section L

Low power micro-photographs of complete sections of rootlets of hyacinths, grown in the solutions indicated under high power micro-photographs with corresponding lettering. Magnification 52 diameters.

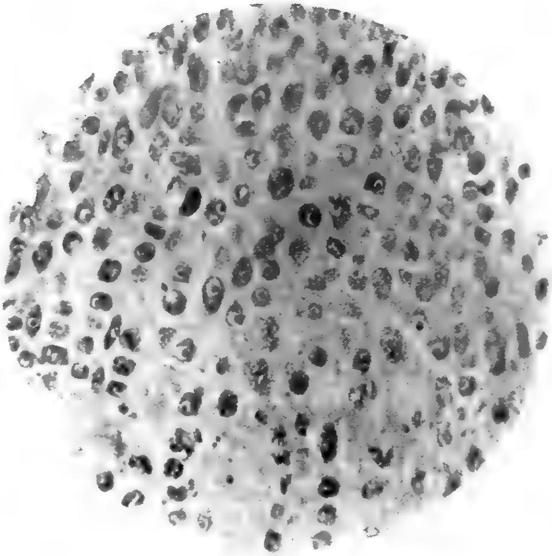


Section A_1.—No. 1. Control

Section B_1.—No. 3. Sodium hydrate (0·0015 M = 0·0060 per cent.)

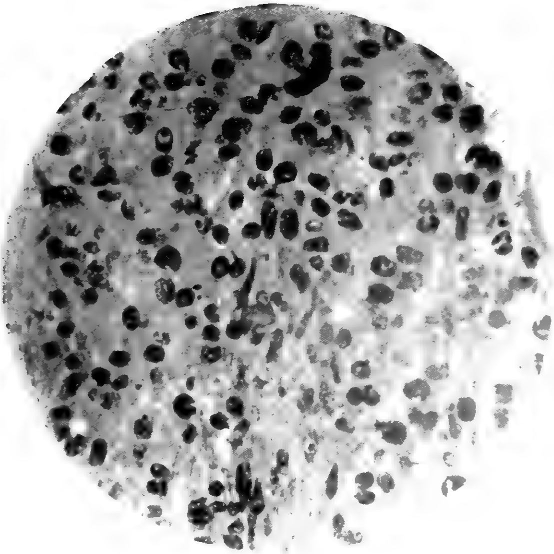


Section C_1.—No 5. Hydrochloric acid (0·0010 M = 0·0036 per cent.)

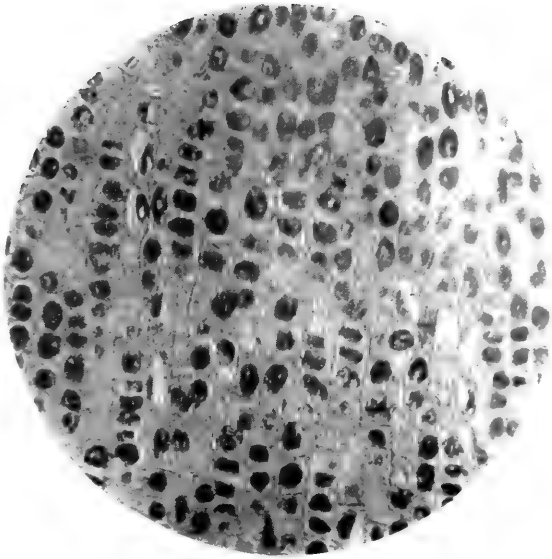


Section D_1.—No. 7. Potassium hydrate (0·0010 M = 0·0056 per cent.)

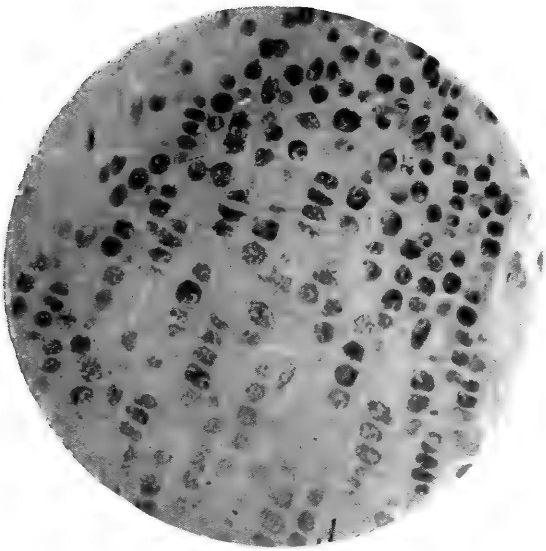
More highly magnified micro-photographs. The magnification is the same in all, viz., 183 diameters. Observe the increase in size of the cells in the acid (Section C_1) and the irregularity of arrangement of cells, frequency of cell divisions, and irregular division figures in the two alkaline Sections B_1 and D_1. The scattered dots in the lower part of D_1 are shortened dot-like chromosomes very irregularly arranged.



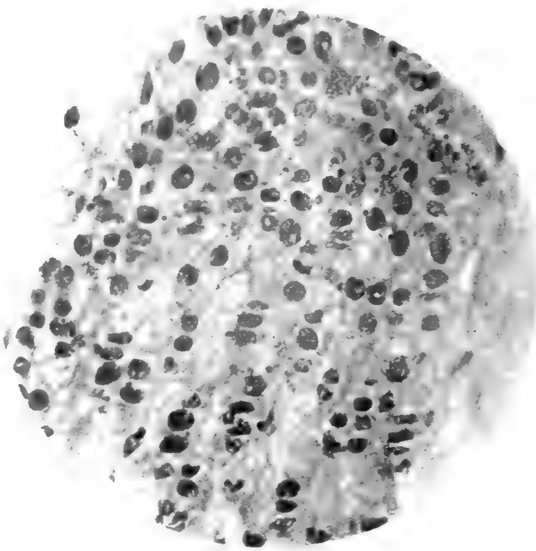
Section E_1.—No. 8. Potassium hydrate
($0{\cdot}0015$ M = $0{\cdot}0084$ per cent.)



Section F_1.—No. 10. Sodium bi-carbonate
($0{\cdot}0030$ M = $0{\cdot}0252$ per cent.)

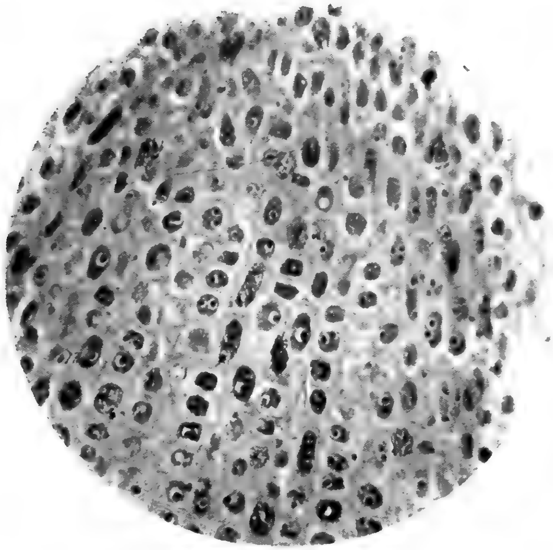


Section G_1.—No. 12. Sodium bi-carbonate
($0{\cdot}0100$ M = $0{\cdot}0840$ per cent.)

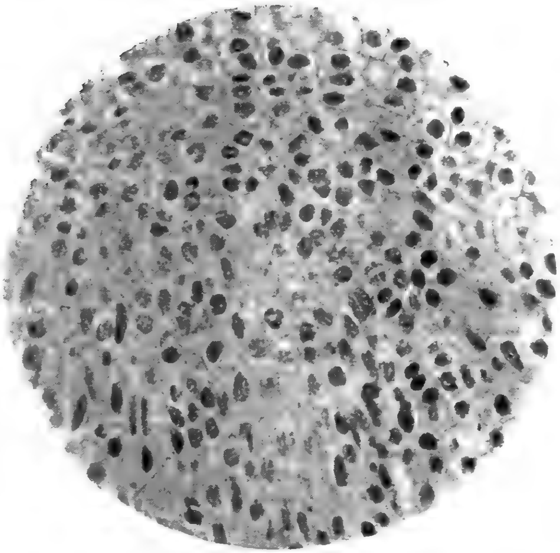


Section H_1.—No 13. Sodium carbonate
($0{\cdot}0015$ M = $0{\cdot}0159$ per cent.)

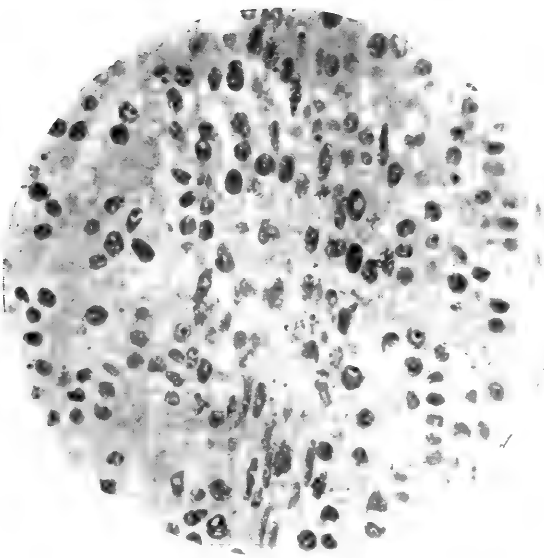
Micro-photographs of same magnification as four previous sections, of rootlets grown in solutions indicated, 183 diameters. Observe the great irregularity in nuclear divisions in the potassium hydrate (Section E_1) and sodium carbonate (Section H_1). The cells are more regularly arranged in the bicarbonate solution sections (Sections F_1 and G_1), but there are many more nuclear dots than in the control (Section A_1).



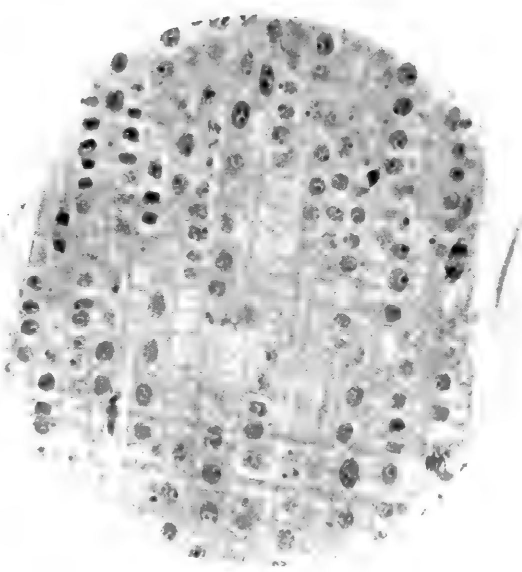
Section I_1.—No. 15. Mono-Sodium phosphate (acid phosphate) (0·0005 M = 0·0060 per cent.)



Section J_1.—No. 17. Mono-Sodium phosphate (acid phosphate) (0·0015 M = 0·0180 per cent.)



Section K_1.—No. 19. Di-Sodium phosphate (alkaline phosphate) (0·0050 M = 0·0700 per cent.)



Section L_1.—No. 20. Di-Sodium phosphate (alkaline phosphate) (0·0100 M = 0·1400 per cent.)

Same magnification as eight previous sections, 183 diameters. Section I_1 is almost normal in the regularity of arrangement of the cells, and infrequency of cell division. In the central portion some of the cells mentioned in the text, with very elongated nuclei, are seen. Compare the frequency of division and irregularity of nuclear figures and masses of chromatin of Section K_1 alkaline phosphate, with the two upper photographs of acid phosphate sections.

AN INVESTIGATION OF THE TOXIC ACTIONS OF DILUTE SOLUTIONS OF THE SALTS OF CERTAIN HEAVY METALS (VIZ.: COPPER, IRON, NICKEL, COBALT, MANGANESE, ZINC, SILVER, AND LEAD) UPON THE *BACILLUS TYPHOSUS*, WITH A VIEW TO PRACTICAL APPLICATION IN THE PURIFICATION OF SHELL-FISH

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(*Received June 17th*, 1908)

Some years after the death of Nägeli (1), in 1891, there was published by Schwendener, a paper which had been found among Nägeli's manuscripts, entitled 'Ueber oligodynamische erscheinungen in lebenden Zellen.' In this paper Nägeli demonstrated the extreme sensitiveness of certain living plants to very minute traces of various metals.

Some years prior to his death Nägeli had observed, during some experiments with Algae, that the latter were killed when placed in distilled water, and although at first he attributed this action to various other causes, he eventually found that it was due to minute traces of copper, which had become dissolved by the water in its passage through the copper still.

Having arrived at this conclusion, Nägeli proceeded to carry out a large number of experiments, using copper coins (two-pfennig pieces, consisting of ninety-five parts of copper, four of tin, and one of zinc) in distilled water, and he even calculated approximately the amount of copper dissolved by the water, viz., 1·3 parts of copper to 100 million parts of water.

He found that this solution was toxic to Spirogyra and that, even when diluted ten times, it would still kill them.

There is no doubt that Nägeli considered the copper to be in a state of solution, although at first he believed the toxic action of the copper to be due to a new force, ‘Isagität,’ and in his original manuscript he uses the word ‘isagische’ in describing it, but later he substituted the word ‘oligodynamische.’

During his investigations which were confined to the action on Spirogyra, Nägeli discovered that minute quantities of other metals, viz., silver, lead, tin, iron and mercury, showed oligodynamic properties similar to copper.

Many investigators have conducted experiments with copper and its salts, since that time, and one of the most important researches is that of Israel and Klingmann (2), which was published in 1897.

Israel and Klingmann carried out a large number of experiments on certain bacteria, including *Bacillus typhosus* and *Bacillus coli*, many animal organisms and Spirogyra; they used copper foil and found that it had a marked toxic effect on all the bacteria they worked with, *B. typhosus* being the most easily killed.

When the copper solutions containing the organisms were incubated at about 40° C., it was found that they were destroyed in half the time necessary to kill them at laboratory temperature.

It was also found that different organisms were differently affected by the copper solution, and although most organisms were killed in a few minutes, some, *e.g.*, Stylonychia, resisted for twenty-four hours, and later experiments by other investigators have shown that some plants not only resist the action of the copper, but even appear to benefit by its presence, under certain conditions.

Locke (3) found that the traces of copper contained in water distilled from or through copper vessels were sufficient to kill Tubifex, a freshwater Annelid.

The fact that the degree of this toxic action is not constant but varies from species to species in plants and animals naturally suggested the practical application of this effect in the purification of water from deleterious organisms.

In 1905 an important paper was published by Moore and Kellerman (4) of the Bureau of Plant Industry of the United States

Department of Agriculture, on a method for destroying or preventing the growth of Algae and certain pathogenic bacteria in water supplies.

These authors claim that with very high dilutions of the salts of copper they were able to kill Algae and certain pathogenic organisms; one part in 50,000,000 killed Algae in watercress beds, 1 in 4,000,000 foul-smelling Anabaena, and 1 in 100,000 killed both cholera and typhoid in four or five hours at laboratory temperature.

Rideal and Baines carried out experiments to control these results, but were not so successful as Moore and Kellerman in disinfecting with such high dilutions, and they could not confirm their results. They came to the conclusion that 1 in 1000 of copper salts was the only safe dilution, but that twenty-four hours in a copper vessel freed the water from *Bacillus typhosus* and *Bacillus coli*.

Kraemer (5) did some very accurate work with a view to testing the efficiency of metallic copper and sulphate of copper in killing *Bacillus typhosus* and *Bacillus coli* in water. He used pure cultures of typhoid and colon bacilli, developed in bouillon for about twenty-four hours, and his results may be summarised for the purposes of the present paper.

1. Filtered tap water prepared by means of a Berkefeld filter attached to a copper spigot, contained a sufficient amount of metallic copper (dissolved from the spigot) to kill *Bacillus typhosus* in less than four hours, and although *Bacillus coli* was not actually killed, its growth was very markedly inhibited, owing to the presence of these minute traces of copper.

2. When strips of copper foil about 15 mm. wide and 18 cms. long were added to two separate series of three different flasks, (*a*) containing triple distilled water, (*b*) filtered water, etc., (*c*) tap water, and to each of which had been added a fresh bouillon culture of typhoid and colon bacilli, respectively, it was found that in four hours all the flasks were quite free from organisms and the solutions remained sterile.

3. When copper sulphate was added to tap water, so that there

was 1 part to 100,000 of water, 97 per cent. of the organisms were destroyed in eight hours. When the strength was reduced so that there was one part of copper sulphate to 1,000,000 parts of water, there was a reduction of 86 per cent.

Owing to the sensitiveness of typhoid and colon bacilli to the influence of copper, as previously shown, it may be inferred that they would have been included in the organisms destroyed.

In 1905 there was published by Fleet Surgeon Bassett-Smith (6) a paper entitled 'Experiments to demonstrate the Germicidal Power of Copper and Copper Salts on Pathogenic and Non-Pathogenic Organisms.'

From this paper only the results of experiments with typhoid bacilli will be quoted.

Bassett-Smith first conducted a series of experiments with different strengths of copper sulphate (viz., 1 in 1,000, 1 in 10,000 and 1 in 100,000) in tap water and distilled water.

It is not apparent from the context of the paper, what is meant by the above dilutions, nor is it possible to determine whether they were prepared from 'gram-molecular' solutions or not. The method of procedure was as follows :—

Ten cubic centimetres of each dilution was inoculated with thirty cubic millimetres of a well-grown broth culture of the organisms; these were kept at room temperature in the dark and sub-cultured at regular intervals.

The result of this series was that in all dilutions with distilled water *B. typhosus* was killed under one hour, but with solutions made with tap water, the bacilli were present at the end of one hour in the 1 in 10,000 solution, but were all dead in three hours; in the 1 in 100,000 solution, the reaction was positive after fifteen hours, but negative in twenty-four hours.

Bassett-Smith comes to the following conclusions, as a result of his experiments to purify contaminated water by means of copper sulphate :—

'The only safe dilution is the low one of 1 in 1000, the solution is best freshly prepared, and at least twenty-four hours' action is

generally necessary. The salt is most efficient when made up with distilled water; and *Bacillus typhosus* is more easily killed than others of the coli group, being destroyed in twelve hours with the 1 in 10,000 dilution.'

In the second part of the paper the results of experiments conducted to discover whether other metals might have destructive or inhibitory power on typhoid and other organisms, are tabulated. Stoppered tubes of iron, copper, zinc, lead, tin and glass were first used, and later on metal boxes capable of containing a much larger quantity of infected water but giving a relatively smaller proportional area of metal to the cubic contents.

Sterilized tap water inoculated with broth cultures of typhoid bacilli was used, and the boxes were kept in the dark at room temperature.

From these experiments it appeared that iron and copper were the most powerfully bactericidal, no growth being obtained twelve hours after treatment; zinc was negative in eighteen hours, lead in forty-eight hours, and the tin and glass remained positive at the end of three days.

It was found, on examination of the boxes, that the surface of the copper and zinc was coated with a thick layer of deposit, and when similar experiments were conducted with the boxes in this state, their bactericidal power was greatly decreased, the solution in the copper box still containing bacilli at the end of three days. When the insides of the boxes were thoroughly cleaned, the action again became normal.

Finally, solutions of granulated sulphate of iron, 1 in 1,000, 1 in 10,000 and 1 in 100,000, were prepared and their germicidal action tested.

Solution 1 in 100,000 was practically non-effective; 1 in 1,000 and 1 in 10,000 were fatal to *B. typhosus* in under seven hours. 'Practically therefore, sulphate of iron added to water in the proportion of 1 in 10,000 would in less than seven hours free it from typhoid bacilli, but not from other members of the coli group, being almost as effective as the more dangerous copper sulphate solution.'

Present Experiments

In January, 1906, the present series of experiments with salts of several of the heavy metals was commenced in order to ascertain their effect on the *Bacillus typhosus* and then, if feasible, that the results obtained might be applied to the purification of shell-fish from this organism.

In the papers quoted previously none of the writers has exactly stated what is meant when speaking of dilutions of '1 in 1,000,' etc., and so the first step was the preparation of a number of gram-molecular solutions from which the dilutions subsequently used were prepared as required (the ferrous sulphate solution was prepared immediately before being used, as it rapidly becomes oxidised), and thus the exact amount of the salt employed in any particular experiment could be accurately determined. The typhoid cultures used were grown on agar, generally for forty-eight hours, some of the growth obtained being collected on a sterilized loop of platinum wire, and an emulsion of typhoid bacilli was made in 100 c.c. of distilled water.

The different solutions were made with (1) distilled water, (2) tap water, collected after being allowed to run for five minutes, and (3) sea water.

In all the experiments the water had been sterilised in an auto-clave at 110° C. for thirty minutes; and all the Erlenmeyer flasks, Petri dishes and pipettes used were sterilized in the same way.

For determining the number of organisms, 1 c.c. of the respective solutions was taken at intervals with a sterile pipette and added to a sterile test-tube, containing litmus red taurocholate agar which had been previously melted by means of a steam bath, and the tubes cooled down to about 40° C. in a beaker containing water at that temperature.

The contents of the tube when inoculated, were immediately placed in a sterile Petri dish and the latter incubated at about 35° C. When the number of colonies was quite uncountable the result is described in the tables as 'infinite'; when, although there was a large number of colonies the number could have been counted, the result is indicated by the word 'positive' (all 'positive' results below

about 1,000 colonies per c.c. have the number of colonies indicated), and when the plates showed no colonies at all the result is expressed by the word 'negative.'

Series I.—Experiments with Copper Salts

The first series of experiments was done with solutions of copper sulphate, in (*a*) distilled water, (*b*) tap water, which had been allowed to run for five minutes, and (*c*) sea water, and the results are tabulated below :—

I (*a*).—1 cubic centimetre of the typhoid emulsion prepared as above was added to each of the following five flasks :—

(1) Containing 100 c.c. of distilled water.
(2) " " $\frac{1}{1,000}$ M Copper sulphate solution made with distilled water.[1]
(3) " " $\frac{1}{10,000}$ M " " " "
(4) " " $\frac{1}{100,000}$ M " " " "
(5) " " $\frac{1}{1,000,000}$ M " " " "

The flasks were incubated at 35° C., and sub-cultures taken at regular intervals.

Distilled Water

		Copper Sulphate Solutions			
Sub-cultures taken	Control	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$	$\frac{M}{1,000,000}$
At once	Infinite	Infinite	Infinite	Infinite	Infinite
15 minutes after ..	"	Negative	Positive	Positive	"
1 hour after ...	"	"	Negative	Negative	Positive (5 colonies)
2 hours after ...	"	"	"	"	Negative
6 hours after ...	"	"	"	"	"
24 hours after ...	"	"	"	"	"

1. The extreme dilution of these solutions is seen when it is remembered that M strength means 63 parts of copper per 1,000, and therefore, $\frac{M}{1,000}$ means 63 parts in 1 million, and $\frac{M}{1,000,000}$ means 63 parts in 1,000 millions. It may be pointed out that the solutions used throughout are much more dilute than the $\frac{1}{1,000}$, etc., of previous authors, since these most probably mean parts by weight of the crystalline salts and not $\frac{1}{1,000}$, etc., gram-molecular solutions.

It will be seen by the above table that in the copper sulphate solutions all the bacilli were destroyed in one hour with the exception of the 1,000,000th dilution, in which five colonies per c.c. persisted, but even this solution was quite sterile at the end of two hours and remained so.

The number of bacilli on the control plate at the end of twenty-four hours was still 'infinite.'

I (*b*).—This was in all respects similar to I (*a*), with the difference that the solutions were made with sterilised tap water instead of distilled water.

It was noticed that the contents of flasks 2 and 3, *i.e.*, the $\frac{M}{1,000}$ and $\frac{M}{10,000}$ dilutions, had a distinctly blue colour and there was a thick sediment at the bottom of these two flasks.

Tap Water

Sub-cultures taken	Control	Copper Sulphate Solutions $\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$	$\frac{M}{1,000,000}$
At once	Infinite	Infinite	Infinite	Infinite	Infinite
15 minutes after ...	„	Negative	Positive	„	„
1 hour after ...	„	„	„ (14 colonies)	Positive	„
2 hours after ...	„	„	Negative	„	Positive
4 hours after ...	„	„	„	„ (173 colonies)	„
8 hours after ...	„	„	„	Negative	„
24 hours after ...	„	„	„	„	„ (193 colonies)
48 hours after ...	„	„	„	„	Positive (147 colonies)

I (*c*).—This was similar to I (*a*) and I (*b*), excepting that the solutions were made with sterilised sea water, instead of distilled or tap water. A thick precipitate was thrown down in all the flasks, but especially in No. 2. The first two dilutions were distinctly blue.

Sea Water

Sub-cultures taken	Control	Copper Sulphate Solutions $\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$	$\frac{M}{1,000,000}$
At once	Infinite	Infinite	Infinite	Infinite	Infinite
2 hours after ...	,,	Positive	Positive	,,	,,
6 hours after ...	,,	Negative	,,	Positive	,,
24 hours after ...	,,	,,	Negative	Negative	Positive
48 hours after ...	,,	,,	,,	,,	,,
72 hours after ...	,,	,,	,,	,,	,,

The most striking fact in the above series of experiments is the exceedingly small quantity of copper sulphate which was sufficient to kill all the typhoid bacilli in a few hours.

This is especially noticeable in the results obtained with solutions made with distilled water, when a dilution of one in a million of gram-molecular was sufficient to kill all the bacilli in less than two hours. The actual amount of copper in this dilution was 63 parts in 1,000 million parts of water, or roughly one of copper in sixteen million parts of water.

In the solutions made with tap water and sea water the action was still very marked, but less so than in the case of the distilled water. This is probably due to the fact that the tap and sea water both contained other substances, which in some way modified the action of the copper sulphate, thus weakening the solutions.

In this connection it is of interest to remember that Moore and Kellerman (4) have shown, in their paper, the relative decrease of toxicity of copper sulphate solutions, depending on the amount of organic matter present, the amount of carbon dioxide in solution, or the temporary hardness of the water.

Nägeli showed that the oligodynamic action of copper solutions was lessened by the introduction of many insoluble substances, and True and Oglevee have studied the influence of insoluble substances on the toxic action of poisons and have confirmed many of Nägeli's observations.

The manner in which the presence of such adventitious substances, inorganic or organic, in the water to which the copper salt is added, produce their effects in diminishing the toxic action, is not discussed

by previous authors, with the exception of Kraemer, who concluded that the toxicity is due to some salt of copper, which 'is probably in the form of a crystalloid rather than that of a colloid.'

It is, however, fairly obvious that the toxic action depends upon the concentration in the solution of the copper ion, because all salts of copper possess the toxicity, which must, therefore, depend upon the presence of the free kation in the solution and not upon the non-ionized molecule of the copper salts, which is different in each case.

When once it is considered that the toxicity is dependent in all cases upon the concentration of the copper ion in the solution, the varying toxicity, when the copper is added to (*a*) distilled water, (*b*) tap water, (*c*) sea water or (*d*) water containing other added salts or organic matter, becomes easy of explanation. The salts present in the water cause the degree of ionization of the copper to vary. For instance, when distilled water only is present and then copper sulphate is added, at the degree of dilution here concerned, the copper sulphate practically completely ionizes into copper ions and sulphion ions, and therefore the concentration in copper ions is proportional to the total amount of copper sulphate added. But when salts such as the phosphates are present, as in tap water or sea water, the ionization in the solution becomes that of the much more feebly ionized copper phosphate and consequently the concentration in copper ions is no longer indicated by the amount of copper sulphate added, but is reduced correspondingly to the low degree of ionization.

Similarly the addition of apparently inert organic substances, such as cellulose, silk, wool, glue, etc., will produce a like effect by forming feeble combinations or adsorptions with the copper ion and thus reduce its concentration.

Since the toxicity, in the case of salts of the heavy metals, depends on the metallic kation entirely and not on the anion of the particular acid in combination in the salt employed, it was considered sufficient in most cases to test only one salt of each metal. Also, since the amount of added salt is so small, any low degree of toxic action of the anion can be disregarded, and the most convenient and easily accessible salt of the heavy metal can be employed.

Secondly, the results of this series tend to strengthen the theory of those observers who contend that in the case of the copper foil in water, the metal itself is not actually in a state of solution as a colloid in the water, but that it forms salts and that it is the action of these salts which determines the toxicity of the solution; for these concentrations of copper as salt are less than the amount of copper in colloidal solutions.

These experiments with such excessively dilute solutions of copper sulphate appear to show very clearly that copper in the ordinary condition of solution as a salt (that is to say in the ionized condition and not in colloidal solution) is capable of exhibiting all the so-called 'oligodynamic' properties, produced when metallic copper is immersed in water in which living organisms are present.

Accordingly, no foundation is left for the term oligodynamic as applied to copper brought into solution by the latter method, and the results obtained are merely an index of the high toxicity of copper ions upon some forms of living cells.

Attention may here be drawn to the fact that the solutions, containing the typhoid bacilli were incubated at a temperature of 35° to 40° C., and this applies to all other experiments with the exception of the ferrous sulphate and ferric chloride series, in which the flasks were kept at room temperature, *i.e.*, about 15° C. Attention is drawn to this matter, because it was proved by Israel and Klingmann that when the solutions of copper containing the organisms were placed in an incubator at 35° to 40° C. the toxic effects were manifested in one hour, but if the solutions were kept at ordinary temperature the toxic effects were not produced until two hours had elapsed; so that, according to this statement, these experiments were conducted under the most favourable conditions.

Series II.—Experiments with Iron Salts

Experiments with dilutions of gram-molecular solutions of ferrous sulphate and ferric chloride made with (*a*) distilled water, (*b*) tap water, after being allowed to run for five minutes, and (*c*) sea water.

In this series seven flasks were used, and 1 c.c. of the typhoid emulsion was added to each of them :—

(1) Containing 100 c.c. of distilled water.

(2) ” ” $\frac{1}{1,000}$M Ferrous sulphate solution made with distilled water.

(3) ” ” $\frac{1}{10,000}$M ” ” ” ”

(4) ” ” $\frac{1}{100,000}$M ” ” ” ”

(5) ” ” $\frac{1}{1,000}$M Ferric chloride ” ”

(6) ” ” $\frac{1}{10,000}$M ” ” ” ”

(7) ” ” $\frac{1}{100,000}$M ” ” ” ”

The flasks were kept at room temperature, about 15° C. Nos. 2, 3, 5, and 6 had a distinct colour, 2 and 3 being at first a pale green, and 5 and 6 a rusty brown, but in twenty-four hours all the solutions were brownish in colour, due to the oxidation of the ferrous sulphate.

Distilled Water

		Ferrous Sulphate Solutions			Ferric Chloride Solutions		
Sub-cultures taken	Control	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once ...	Infinite	Infinite	Infinite	Infinite	Infinite	Infinite	Infinite
2 hours after ...	”	Positive (314 colonies)	Positive	”	”	Positive	”
6 hours after ...	”	Negative	Negative	Negative	Negative	Negative	”
24 hours after ...	”	”	”	Positive	Negative	”	Positive
48 hours after ...	”	”	”	Negative	Negative	”	Negative

The above table shows that both with the ferrous sulphate and ferric chloride solutions, the 1 in 1,000 and 1 in 10,000 gram-molecular dilutions completely destroyed the bacilli in less than six hours, with the flasks at laboratory temperature.

In both cases the 1 in 100,000 solutions still contained over 1,000 bacilli per cubic centimetre at the end of twenty-four hours, but were quite free in less than forty-eight hours.

II (*b*).—This was similar to II (*a*), with the difference that the solutions were prepared with tap water instead of distilled water. Flasks Nos. 2, 3, 5, and 6 have a more distinct colour than in the

previous series, and the ferrous sulphate solutions have turned yellow. There is a precipitate thrown down to a slight degree in all flasks, but more marked in 2 and 3.

Tap Water

Sub-cultures taken	Control	Ferrous Sulphate Solutions $\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$	Ferric Chloride Solutions $\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once ...	Infinite	Infinite	Infinite	Infinite	Infinite	Infinite	Infinite
2 hours after ...	"	Positive	Positive	"	Positive	Positive	"
4 hours after ...	"	"	"	"	"	"	"
8 hours after ...	"	Negative	Negative	Positive	Negative	Negative	Positive
24 hours after...	"	"	"	"	"	"	"
48 hours after...	"	"	"	" (227 colonies)	"	"	" (335 colonies)

It will be noticed on comparing the above table with II (*a*) that the action of the solutions made with tap water was slower than that made with distilled water, and the 1 in 100,000 solutions were still positive at the end of forty-eight hours.

II (*c*).—Similar in details to II (*a*) and II (*b*) except that the solutions were made with sea water instead of distilled or tap water. There was a thick brownish deposit in all the flasks, after they had been incubated, with the exception of the control, in which the deposit was less marked and not coloured to any extent.

Sea Water

Subcultures taken	Control	Ferrous Sulphate Solutions $\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$	Ferric Chloride Solutions $\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once	Infinite	Infinite	Infinite	Infinite	Infinite	Infinite	Infinite
2 hours after ...	"	Positive	Positive	Positive	Positive	Positive	Positive
5½ hours after	"	Negative	"	"	" (17 colonies)	"	"
24 hours after...	"	"	Negative	"	Negative	Negative	"
48 hours after...	"	"	"	"	"	"	"

It is seen from this table that the toxicity of the solutions made with sea water is distinctly less than was the case with the distilled water, and slightly less than that of the solutions made with tap water. This is probably explained in the same way as in the case of the copper sulphate solutions, and is no doubt due to salts, etc., in the

water which modify the action of the solutions—in the latter two cases by lowering the ionization as previously described.

In this series the marked toxicity of the solutions of salts of iron is strikingly demonstrated. This is all the more remarkable, firstly, because this series was conducted at laboratory temperature, *i.e.*, 15° C. instead of about 40° C. as was the case with Series I (it has been previously shown that the incubation of the solutions greatly increases their killing power), and secondly, because salts of iron are not poisonous in the ordinary sense of the word.

It will further be seen that the action of the two salts was very similar in each series, the ferrous sulphate solutions being slightly more toxic than the ferric chloride.

This is rather remarkable, as the ferrous sulphate always became converted into a ferric salt after the lapse of a few hours, but the result may be due to increased ionization of the ferric sulphate formed.

It is interesting to compare these results with those obtained by Bassett-Smith in experiments with water infected with typhoid bacilli and contained in (*a*) an iron tube, and (*b*) an iron box of much larger capacity.

In the iron tube all the bacilli were killed in less than twenty-four hours, and in the iron box in less than 18 hours; but in the latter case the solution was not so heavily infected with bacilli as in the former. In both cases the water, at the end of twenty-four hours, was quite a rusty colour, due to the formation of oxide of iron.

The results of this series are important for several reasons. In the first place, although the toxicity of the salts of iron is less than that of the copper sulphate solutions, it is still sufficient to be of great practical importance, and whereas the safe dose for human beings has still to be determined in the case of copper salts, it is well known that not only are salts of iron not deleterious to man, but that many of them are even beneficial and are used medicinally. This fact is also of importance in regard to the purification of drinking water by addition of salts of iron or by storage in iron tanks, and also in the purification of suspected shell-fish.

It is, moreover, possible to employ iron in the manufacture of

tanks for water purification when the use of copper could not be entertained owing to its great cost.

The results of the experiments made with these salts of iron in attempting to purify Anodons infected with *B. typhosus* are tabulated in Part II of this paper.

Series III.—Experiments with Nickel and Cobalt Salts

Seven flasks were used to contain the different solutions, which were made with distilled water.

The 1 in 1,000 solutions were coloured green and red respectively, but the others were colourless.

One c.c. of the typhoid emulsion was added to each of the flasks and they were then incubated at 40° C.

		Cobalt Chloride Solutions			Nickel Chloride Solutions		
Sub-cultures taken	Control	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once ...	Infinite	Infinite	Infinite	Infinite	Infinite	Infinite	Infinite
1 hour after ...	,,	,,	,,	,,	,,	,,	,,
3 hours after ...	,,	Positive	,,	,,	Positive	,,	,,
8 hours after ...	,,	,,	Positive	,,	,,	Positive	,,
24 hours after...	,,	Negative	,, (107 colonies)	Positive	Negative	,, (203 colonies)	Positive
48 hours after...	,,	,,	Negative	,,	,,	Negative	,,

It will be seen from this table that although cobalt and nickel chlorides have a toxic action on typhoid bacilli, it is much less marked than in the case of the copper or iron salts and takes a much longer time to manifest itself.

This is interesting because these metals belong to the group of heavy metals, and yet possess a comparatively low toxicity.

Taking the 'distilled water' series in each case and comparing the results it is seen that whereas the copper sulphate solutions, including the 1 in 100,000, were clear at the end of an hour, and the first two dilutions of the ferrous sulphate and ferric chloride experiments were clear in less than six hours, *at room temperature* the corresponding cobalt and nickel solutions still contained over 1,000 organisms per c.c. in each case at the end of eight hours, and the 1 in 10,000 solutions were not clear at the end of twenty-four hours.

As the toxic effects would have taken still longer to manifest

themselves in solutions made with tap or sea water, this series was not continued, as it was considered that the results would be of no practical value, even under the most favourable conditions.

Series IV.—Experiment with Manganese Salts

The solutions were made with distilled water and were incubated at about 40° C.

To each of four flasks was added 1 c.c. of the typhoid emulsion, prepared as in the previous experiments.

		Manganese Chloride Solutions		
Sub-cultures taken	Control	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once … …	Infinite	Infinite	Infinite	Infinite
1 hour after …	”	”	”	”
4 hours after …	”	”	”	”
8 hours after …	”	Positive	”	”
24 hours after …	”	Negative	Positive (79 colonies)	Positive
48 hours after …	”	”	Negative	” (27 colonies)

It is seen that although manganese chloride has bactericidal power, it is much feebler than in the case of the copper and iron salts.

Series V.—Experiments with Zinc Salts

Four flasks were used as in the previous experiment and the solutions were all made with distilled water and incubated at about 40° C. One c.c. of the emulsion of typhoid bacilli was added to the contents of each flask.

		Zinc Sulphate Solutions		
Sub-cultures taken	Control	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once … …	Infinite	Infinite	Infinite	Infinite
1 hour after … …	”	Positive	”	”
4 hours after … …	”	”	Positive	”
8 ” ” … …	”	” (507 colonies)	”	Positive
24 ” ” … …	”	Negative	” (43 colonies)	”
48 ” ” … …	”	”	Negative	” (403 colonies)

These results were not exactly what were expected, having regard to those obtained by Bassett-Smith with water infected with typhoid bacilli and placed in (*a*) a zinc tube, and (*b*) a zinc box.

In the former case the water was cleared of bacilli in eighteen hours, and in the latter in less than twenty-four hours, the water in this case being more heavily infected than was the case with the zinc tube.

As against this, however, the same observer conducted a special experiment with strips of zinc foil, to see if they would be able to clear water infected with typhoid bacilli, as the copper foil had done. Strips of zinc foil, having a superficial area of 50 square centimetres were added to a flask containing 100 c.c. of sterilized tap water, and the latter was infected with a broth culture of typhoid bacilli and kept at room temperature in the dark.

'Up to forty-eight hours this did not seem to be effective against any of the tested organisms.'

Furthermore, it must not be forgotten that the emulsion of typhoid bacilli used in all these experiments contained very many more bacilli per cubic centimetre than did the broth cultures used in the experiments cited above.

(*b*) The above series was repeated with solutions made with tap water instead of distilled water, and the flasks were kept at room temperature.

		Zinc Sulphate Solutions		
Sub-cultures taken	Control	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once	Infinite	Infinite	Infinite	Infinite
2 hours after	,,	,,	,,	,,
4 ,, ,, ...	,,	Positive	,,	,,
8 ,, ,, ...	,,	,,	,,	,,
24 ,, ,, ...	,,	Negative	Positive	,,
48 ,, ,, ...	,,	,,	,,	Positive

It will be seen from the above table that with the solutions made with tap water and kept at room temperature (about 15° C.) the toxic action was even less marked.

SERIES VI.—EXPERIMENTS WITH SILVER SALTS

Experiments were carried out with different strengths of silver nitrate made with (*a*) distilled water and (*b*) tap water, after being allowed to run for five minutes.

(*a*) Four flasks were used, and the solutions were made with distilled water and incubated at 40° C.

One cubic centimetre of the typhoid emulsion was added to each of the flasks.

Sub-cultures taken	Control	Silver Nitrate Solutions $\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once	Infinite	Infinite	Infinite	Infinite
2 hours after	,,	Negative	Negative	Negative
4 hours after	,,	,,	,,	,,
6 ,, ,, ...	,,	,,	,,	,,
24 ,, ,, ...	,,	,,	,,	,,
48 ,, ,, ...	,,	,,	,,	,,

The results of this series were very definite and proved the silver nitrate to have a very decided germicidal action on the *B. typhosus.* Comparing the above table with that showing the results of the copper sulphate solutions made with distilled water, it is seen that the action of the silver nitrate is practically as great as that of the copper sulphate.

(*b*) This series was the same as (*a*), except that the solutions were made with sterilised tap water collected as described before.

It was immediately noticed that the solutions were inclined to be milky, the 1 in 1,000 and the 1 in 10,000 decidedly so, and the 1 in 100,000 faintly milky.

This was of course due to the precipitation of the silver salt by the chlorides present in the water; the result being the formation of silver chloride which is practically insoluble in water.

Sub-cultures taken	Control	Silver Nitrate Solutions $\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once	Infinite	Infinite	Infinite	Infinite
2 hours after	,,	Negative	Positive	,,
4 ,, ,, ...	,,	,,	,,	,,
6 ,, ,, ...	,,	,,	Negative	,,
24 ,, ,, ...	,,	,,	,,	Positive
48 ,, ,, ...	,,	,,	,,	,,

It is noticed that in the 1 in 1,000 solution there was enough silver nitrate left after all the chlorides had been precipitated[1] to still make the solution strongly germicidal.

1. The Liverpool Water Supply is almost free from chlorides.

In the case of the 1 in 10,000 solution the result either indicated (i) that there was still enough silver nitrate left after the precipitation of the chlorides to make the solution sufficiently toxic to kill the typhoid bacilli in less than six hours, or (ii) that if all the silver nitrate was precipitated as chloride, there must have been a sufficient quantity of this so-called insoluble salt dissolved in the water to produce the toxic effect.

In the 1 in 100,000 solution, the toxicity was very greatly diminished by making it with tap water instead of with distilled water.

Series VII.—Experiments with Lead Salts

This series was done with solutions of lead nitrate made with (*a*) distilled water and (*b*) tap water, collected after being allowed to run for five minutes.

Four flasks were used in each case, and were incubated at about 40° C. One cubic centimetre of the emulsion of typhoid bacilli was added to each of the flasks.

(*a*) Solutions made with distilled water :

		Lead Nitrate Solutions		
Sub-cultures taken	Control	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once	Infinite	Infinite	Infinite	Infinite
2 hours after	,,	Negative	Positive	,,
4 hours after	,,	,,	Negative	Positive
6 ,, ,, ...	,,	,,	,,	,,
24 ,, ,, ...	,,	,,	,,	,, (879 colonies)
48 ,, ,, ...	,,	,,	,,	,, (53 colonies)

(*b*) Solutions made with tap water, collected as previously :—

		Lead Nitrate Solutions		
Sub-cultures taken	Control	$\frac{M}{1,000}$	$\frac{M}{10,000}$	$\frac{M}{100,000}$
At once	Infinite	Infinite	Infinite	Infinite
2 hours after	,,	Negative	,,	,,
4 ,, ,, ...	,,	,,	Positive	,,
6 ,, ,, ...	,,	,,	,,	,,
24 ,, ,, ...	,,	,,	,,	Positive
48 ,, ,, ...	,,	,,	,,	,,

It is seen that the lead nitrate solutions both with distilled and tap water were decidedly toxic to the bacilli, but that the solutions made with distilled water were much stronger and quicker in their action than those made with tap water.

Here it is interesting to note the result of experiments by Bassett-Smith, done with metallic lead, to see whether solutions containing *B. typhosus* could be rendered sterile by being placed in (*a*) a lead tube, and (*b*) a lead box.

In both cases the solutions still contained many organisms at the end of forty-eight hours, and it could not be said that the lead had any germicidal action.

On the other hand, Nägeli did experiments with lead, and was satisfied that it possessed ‘ oligodynamic ’ properties similar to copper.

Conclusions to Part I

The following conclusions were arrived at as the result of the foregoing experiments :—

(1) All the salts tested manifest a decided toxic action to typhoid bacilli, but there is a great difference in degree, shown in the case of the stronger solutions by the time taken by the corresponding solutions of the various salts in clearing the water from bacilli ; and in the failure of the more dilute solutions of the less toxic salts to clear within the limit of forty-eight hours.

(2) The toxic action is most marked when the solutions are prepared with distilled water, and are incubated at a temperature of 35° to 40° C.

(3) The toxic action of the solutions made with tap water and sea water is much less than in the case of those made with distilled water, and this diminution is due to the fact that the tap and sea water both contain other substances which modify the action of the salts employed—probably by lessening the ionization.

(4) The copper sulphate and silver nitrate solutions possess the greatest toxicity; the lead nitrate, ferrous sulphate, and ferric chloride solutions being next in order of toxicity ; whilst the zinc sulphate, nickel and cobalt chlorides and manganese chloride solutions take a

much longer time before the corresponding solutions are free from bacilli.

(5) The toxicity of water to which either copper foil or certain other metals (*e.g.*, silver, iron, tin, lead, etc.) have been added is probably due to a solution of some salt of that metal, and the so-called oligodynamic action of the solution is due to the presence of the ions of this salt, and not to the metal itself.

Part II

Experiments on Purification of Shell-fish

Having determined by means of the experiments detailed in Part I the salts most likely to prove successful in purifying infected shell-fish, an endeavour was made to purify Anodons (freshwater mussels) which had previously been strongly infected with typhoid bacilli.

It was first necessary to discover the actions, if any, of the solutions it was proposed to use on the Anodons themselves, and a series of experiments was first carried out in each case with this end in view.

In these experiments a number of glass dishes was used, in which the Anodons were placed in known quantities and strengths of various solutions.

The Anodons used were of medium size and good vitality, and all the glass dishes were very thoroughly cleaned after each series of experiments.

When it was required to infect the Anodons with typhoid bacilli the process was carried out as follows :—

An emulsion of typhoid bacilli was first made in 100 c.c. of sterilized tap water, exactly as in the experiments in Part I, with the exception that tap water was used instead of distilled water.

This emulsion was made up to two litres with tap water (collected after being allowed to run for five minutes) in one of the glass dishes, and into this solution twelve Anodons were placed and left for at least twenty-four hours.

At the end of this time they were taken out as required, and put, without any preliminary cleansing, into the various solutions.

The solutions containing the Anodons were kept at laboratory temperature, and in the case of the silver nitrate experiments they were kept in the dark, as it was found that, unless this precaution was taken, the silver became reduced and the solutions turned dark brown.

The determination of the number of organisms in different parts of the Anodons before and after treatment with the solutions was made by passing a sterile capillary tube (*a*) into the stomach, (*b*) into the rectum, and (*c*) over and among the gills.

In each case the water in the capillary tube was added to a sterile test-tube, containing litmus red taurocholate agar which had previously been melted and kept at 40° C. by means of hot water in a beaker, and the contents were then placed at once in a sterile Petri dish and incubated at about 40° C.

The average capacity of the capillary tubes was 0·05 c.c., but they varied slightly in size.

Before these cultures could be made it was necessary to open the shell of the Anodon, and to do this the anterior and posterior adductor muscles had first to be cut through with a sharp knife.

When it was possible to slightly open the shell this could be done by passing the knife along inside, keeping close to the shell, and first cutting the anterior and then the posterior adductor or *vice versa.*

When, however, it was impossible to open the shell even a little, it was first necessary to cut the edge with a sharp pair of scissors, when the knife could be used as before.

The knife and scissors used were carefully sterilized after each experiment, as were also the hands of the operator.

Metallic and Colloidal Copper

I. Experiments with (1) Anodons and (2) Anodons infected with typhoid bacilli, in (*a*) tap water containing a piece of bright copper foil 15 cms. by 12 cms., (*b*) a solution of colloidal copper sulphate.

As a preliminary to this series, a solution of colloidal copper was prepared by passing an electric current through distilled water, using

copper electrodes, which were so arranged as to form an electric arc under the water.

The current was cut off at frequent intervals to prevent the solution from getting hot, as when this occurs the copper becomes coagulated and precipitated, and then appears as flaky masses in the solution. Altogether nearly three litres of this solution, which is of a slightly greenish colour, were prepared.

The next step was to estimate the amount of copper in the colloidal solution, and this was done as follows :—

Five hundred cubic centimetres of the solution were evaporated to dryness in a clean glass evaporating dish, the residue being dissolved in about twelve drops of strong nitric acid, diluted with a little water, and this solution was again evaporated to dryness.

This residue was dissolved in distilled water containing a drop of hydrochloric acid.

The solution was transferred to a clean glass beaker (the evaporating dish being twice washed out with a little distilled water to dissolve any residue), heated to boiling, precipitated as hydrate, and filtered and weighed in a Gooch crucible.

Weight of Gooch crucible alone	6·0960 grammes
" " containing residue	6·1098 grammes
" " weight of CuO	0·0138 "
" " " Cu	0·0110 "

This represented the amount of copper in 500 c.c. of the colloidal copper solution, and ∴ the amount in one litre was 0·0220 grammes.

This represents 1·1 grammes of copper in 50,000 c.c.

A series of experiments was next done to discover the degree of toxicity of this solution.

Four Erlenmeyer flasks were taken, as under, and to each of them was added 1 c.c. of typhoid emulsion, prepared as in the experiments in Part I.

The flasks were left at laboratory temperature, and sub-cultures made at regular intervals.

(1) Containing 100 c.c. of distilled water.
(2) „ 100 c.c. of the colloidal copper solution.
(3) „ 10 c.c. „ „ made up to 100 c.c. with distilled water.
(4) „ 1 c.c. „ „ „ „

Sub-cultures taken	Control	Colloidal Copper Solutions Original	1 in 10	1 in 100
At once ...	Infinite	Infinite	Infinite	Infinite
2 hours after ...	„	Negative	Negative	Negative
4 „ „	„	„	„	„
8 „ „	„	„	„	„
24 „ „	„	„	„	„

It will be seen from the above table of results that the colloidal copper solution had a marked toxic action on typhoid bacilli, and even when diluted 100 times it killed them in less than two hours at laboratory temperature.

The experiments with dilutions exceeding 1 in 1,000,000 of (*a*) copper in the ionic form, as present in dilute solution of copper sulphate, and (*b*) of colloidal copper, as in the above experiment, agree in giving complete removal of the bacilli within the two hours time.

It is almost impossible to follow with accuracy the toxic properties beyond these limits, and hence we may for the present regard the two forms of copper as being approximately equally toxic, and both as lying at the upper limit of high toxicity.

It is not at all easy to determine the exact state of the copper in this colloidal solution, for although, since the classical experiments of Graham, it has been customary to apply the name colloidal to those substances which will not pass through animal membranes, more recent researches have shown that there are two sub-classes of colloidal mixtures—the one having the characteristic properties of true solutions, *i.e.*, possessing osmotic pressure, diffusibility, and usually a limited solubility at some temperature: the other being without these properties, and being in the nature of macroscopic or microscopic suspensions.

The fact that this colloidal copper solution possesses the property

of permeating colloids, as the cell wall and the organized contents of the cell, thereby producing marked disturbances in the cell and exerting its toxic action, tends to show that it in some ways resembles the crystalloids.

Whatever may be the true nature of the solution, it is certain that its toxicity is not merely due to this 'colloidal' state, for Roaf and Whitley (7) have shown that whereas colloidal silver prepared in the above manner was exceedingly toxic to tadpoles, a solution of colloidal platinum had absolutely no effect upon them.

It must also be borne in mind that the bacilli present in the solution are producing carbonic acid and weak organic acids which are capable of acting upon the colloidal copper and causing it to pass from the colloidal form to true solution in the ionic condition. When regard is paid to the excessively minute quantity of copper present in the solution, it is obvious that the bacterial products would be quite sufficient to cause the copper to form a salt, become ionized, and act in the same way as the excessively dilute solutions of salts of copper used in the former experiment in Part I with copper sulphate.

Further, when once the colloidal copper entered the cells it would meet there with substances capable of converting it into a copper salt; so that whether the copper be present in the solution as a colloid or as an ion it would, in the end, produce its effect as copper ion; and the results obtained with copper sulphate, when present in so high a dilution as one part of copper in 10,000,000, show that copper, qua copper ion, is capable of producing all the effects obtained with colloidal solutions, so that no peculiar 'oligodynamic properties' or 'colloidal condition' need be assumed in order to explain the results obtained.

Having determined the amount of toxicity of the colloidal copper solution, the experiments with Anodons were now proceeded with in solutions containing different forms of copper.

Two Anodons were placed in each of the following dishes to

determine the action of the different solutions on them. The dishes were kept at laboratory temperature.

(1) Containing 2 litres of tap water, collected after being allowed to run for 5 minutes.
(2) „ 2 „ „ with a piece of bright copper foil.
(3) „ 1 litre colloidal copper solution.
(4) „ 2 litres $\frac{M}{2,000}$ copper sulphate solution, made with tap water.
(5) „ 2 „ $\frac{M}{5,000}$ „ „ „
(6) „ 2 „ $\frac{M}{10,000}$ „ „ „

It was noticed that there was soon a good deal of mucus in the solutions.

	24 hours	48 hours	72 hours	1 week	2 weeks	4 weeks
1st dish	2 alive	2 alive	2 alive	2 alive	2 alive	2 alive
2nd „	2 „	2 „	2 „	2 „	2 „	2 „
3rd „	2 „	2 „	2 „	2 „	2 „	2 „
4th „	1 dead	1 dead	2 dead	—	—	—
5th „	2 alive	2 alive	2 alive	1 alive	2 dead	—
6th „	2 „	2 „	2 „	2 „	2 dead	—

It will be seen from this table that the copper foil and colloidal copper solutions did not visibly affect the Anodons in four weeks. This is probably due to the fact that the Anodons secreted a good deal of mucus, which in some way precipitated the copper in the solutions and thus stopped or greatly modified its action.

It was noticed that the copper foil rapidly lost its lustre and became dull; and in the colloidal copper solution the mucus at the bottom of the dish was tinged green, whilst the water itself was quite clear.

In the case of the copper sulphate solutions, although the Anodons secreted mucus on account probably of the irritation from the solutions, and thus, no doubt, modified their toxicity somewhat, the solutions still remained too powerful for them, and even in the 1 in 10,000 dilution both the Anodons were dead in less than two weeks.

The dead Anodons from the two strongest copper sulphate solutions were distinctly blue in colour, due to the staining with the salt.

It is interesting to remember in connection with the above experiments that Herdman and Boyce (8) found in their experiments with oysters that salts of copper always had a deleterious effect on the oysters themselves.

A series of experiments was next done with Anodons which had been strongly infected by being placed in a solution containing typhoid bacilli (prepared as previously described) for twenty-four hours.

Three of these infected Anodons were placed in each of the following dishes :—

(1) Containing 2 litres of tap water, collected as usual.
(2) ,, a piece of bright copper foil, 15 cm. × 12 cm., in tap water collected as usual.
(3) ,, 1 litre of colloidal copper solution.
(4) ,, 2 litres of $\frac{M}{10,000}$ copper sulphate solution made with tap water.
(5) ,, 2 ,, $\frac{M}{100,000}$,, ,, ,, ,,

Cultures were made at intervals from the stomach, gills, and rectum (indicated in the following tables, S., G., and R. respectively), and the results are shown below :—

Cultures taken	Control			Water with copper foil			Colloidal copper solution		
	S.	G.	R.	S.	G.	R.	S.	G.	R.
At once ...	312	563	233	—	—	—	—	—	—
24 hours after	—	—	—	211	294	178	53	107	21
48 ,, ,,	243	471	187	147	207	99	47	127	34

Cultures taken	Control			$\frac{M}{10,000}$ Copper Sulphate			$\frac{M}{100,000}$ Copper Sulphate		
	S.	G.	R.	S.	G.	R.	S.	G.	R.
At once ...	312	563	233	—	—	—	—	—	—
24 hours after	—	—	—	43	89	52	163	419	137
48 ,, ,,	243	471	187	47	111	23	143	352	103

It will be seen from the above table of results that only the colloidal copper solution and the 1 in 10,000 copper sulphate showed any marked power in dealing with the infected Anodons, that the maximum effect of these solutions was manifested in twenty-four hours, and that the next twenty-four hours' treatment did not seem to have any further action in diminishing the number of the bacilli.

This seems to show that the different solutions practically lost their toxicity in twenty-four hours, as far as the bacilli were concerned,

and this was probably due to the secretion of mucus by the Anodons and the consequent precipitation of the copper salts.

As regards the Anodons themselves, in the 1 in 10,000 solutions, this explanation is hardly sufficient, for if this solution lost all its toxicity in twenty-four hours, the reason for the death of the Anodons in less than fourteen days is not apparent.

It is possible that the Anodons were so injured by the solution in the first twenty-four hours as to be unable to recover from the effects, and it is certain that the toxicity of this solution to typhoid bacilli was much diminished when the Anodons were present in it.

Series II.—Experiments with Iron Salts

Experiments to discover the action of ferrous sulphate and ferric chloride solutions on (1) Anodons, and (2) Anodons infected with typhoid bacilli.

(*a*) A series of dishes was again used containing different solutions, and in each were placed two Anodons.

(1) Containing 2 litres of tap water collected as usual.

(2)	,,	2	,,	$\frac{M}{1,000}$	Ferrous sulphate	solution	made with	tap water.
(3)	,,	2	,,	$\frac{M}{10,000}$	,,	,,	,,	,,
(4)	,,	2	,,	$\frac{M}{1,000}$	Ferric chloride		,,	,,
(5)	,,	2	,,	$\frac{M}{10,000}$	,,	,,	,,	,,

The result of the above series was that at the end of five weeks all the Anodons were still alive, and apparently quite healthy; and whereas at the time of starting the series one or two seemed a little sickly, these revived in the solutions and seemed to benefit by the presence of iron salts in the water.

These results also coincide with the observations of Herdman and Boyce, who found that salts of iron seemed to have a favourable action on oysters.

(*b*) A series was now done with infected Anodons, three of

which were placed in each of five dishes containing solutions similar to the above, and the results are tabulated below :—

Sub-cultures	Control			$\frac{M}{1,000}$ Ferric Chloride			$\frac{M}{10,000}$ Ferric Chloride		
	S.	G.	R.	S.	G.	R.	S.	G.	R.
At once									
24 hours after	—	—	—	0	54	0	70	93	49
48 ,, ,,	251	263	153	0	53	0	17	127	54

Sub-cultures	Control			$\frac{M}{1,000}$ Ferrous Sulphate			$\frac{M}{10,000}$ Ferrous Sulphate		
	S.	G.	R.	S.	G.	R.	S.	G.	R.
At once ...									
24 hours after	—	—	—	0	7	0	61	104	53
48 ,, ,,	251	263	153	0	0	0	10	57	11

It is seen from the above table that ferrous sulphate and ferric chloride solutions both had a decided action in clearing the Anodons from the bacilli, and this was especially noticeable in the case of the ferrous sulphate solutions which were freshly prepared. It is remarkable that these salts should have such a decided action on the Anodons infected with typhoid bacilli, but the explanation is probably to be found in the fact that the iron solutions were not only not irritating to the Anodons but were even beneficial, and so they did not attempt by secreting mucus or in other ways to nullify the toxic action of the solutions.

There was a certain amount of mucus secreted by the Anodons, however, but not nearly so much as in the copper solutions, and it was of a ‘ rusty ’ colour, due to the iron.

These results were considered satisfactory, as these salts could easily be used on a large scale for purifying oysters; for it has already been shown by Herdman and Boyce that they are likewise benefited by the presence of iron salts in the water. Again, it was noteworthy that the chief action took place in the first twenty-four hours, and this fact was the basis of another series of experiments.

(*c*) Another batch of infected oysters was taken and three of them placed in each of a series of glass dishes :—

(1) Containing tap water collected as usual.

(2) ,, $\frac{M}{1,000}$ Ferric chloride solution made with tap water.

(3) ,, $\frac{M}{1,000}$ Ferrous sulphate ,, ,, ,

An Anodon from (1) was opened immediately, and the cultures showed stomach 314, gills 517, and rectum 271 colonies respectively.

At the end of fifteen hours the Anodons from each dish were taken and rapidly rinsed in clean water, and placed respectively in three other dishes containing fresh solutions of the same strength, and were left for ten hours longer.

Cultures were then taken from one Anodon out of each dish, and the result was as follows :—

Cultures	Control			$\frac{M}{1,000}$ Ferric Chloride Solution			$\frac{M}{1,000}$ Ferrous Sulphate Solution		
	S.	G.	R.	S.	G.	R.	S.	G.	R.
At once ...	314	517	271	—	—	—	—	—	—
25 hours after	207	384	159	0	0	0	0	0	0

This change into fresh solutions made the action much more marked, and both the Anodons tested from the iron solutions at the end of twenty-five hours were free from typhoid bacilli.

This action of ferrous sulphate and ferric chloride may possibly prove of great practical value in the treatment of suspected oysters, and a series of experiments will be carried out, shortly, using sea-water solutions of iron salts and oysters infected with typhoid bacilli, with this end in view.

Series III.—Experiment with Silver Salts

III. This series was started to find out whether it would be possible to use silver nitrate solutions to purify infected Anodons.

A series of three dishes was used, and they were kept at laboratory temperature in the dark.

It was noticed that in dishes 2 and 3 the solutions were milky, due to the precipitation of the chlorides in the water as silver chloride.

Two Anodons were placed in each of the following :—

(1) Containing tap water, collected as usual.

(2) ,, $\frac{M}{1,000}$ silver nitrate solution made with tap water.

(3) ,, $\frac{M}{10,000}$,, ,, ,, ,,

In four days it was noticed that the Anodons were sickly in

dishes 2 and 3, and they were all dead in these two dishes six days after being placed in the solutions.

The Anodons in dish 1 remained healthy.

No attempt was made to purify infected Anodons with these solutions, which had proved so harmful to the Anodons themselves.

Series IV.—Experiments with Zinc and Lead Salts

IV. This series was conducted to discover the action of zinc sulphate and lead nitrate on Anodons, and, if feasible, to try and purify infected Anodons with solutions of these salts.

A series of five glass dishes was used, and into each of them two Anodons were put.

(1) contained 2 litres of tap water collected as before.

(2) " 2 " $\frac{M}{1,000}$ zinc sulphate solution made with tap water.

(3) " 2 " $\frac{M}{10,000}$ " " " "

(4) " 2 " $\frac{M}{1,000}$ lead nitrate solution made with tap water.

(5) " 2 " $\frac{M}{10,000}$ " " "

The Anodons in dishes 2 and 4 were all dead in less than one week, and so it was concluded that these solutions could not have any practical application.

The Anodons in dishes 1, 3, and 5 were still living, and apparently healthy after three weeks.

There was a fair amount of mucus in all the dishes, but less in the control.

Another batch of Anodons was now infected with typhoid bacilli, and three of them were placed in each of the following dishes :—

(1) Containing 2 litres of tap water, collected as before.

(2) " 2 " $\frac{M}{10,000}$ zinc sulphate solution, made with tap water.

(3) " 2 " $\frac{M}{10,000}$ lead nitrate " " "

Sub-cultures were taken from the stomach, gills, and rectum of Anodons from each of the above dishes at certain intervals, as shown in the following table :—

Sub-cultures	Control			$\frac{M}{10,000}$ Zinc sulphate solution			$\frac{M}{10,000}$ Lead nitrate solution		
	S.	G.	R.	S.	G.	R.	S.	G.	R.
At once ...	453	719	327						
24 hours after ...				179	435	192	74	109	31

Both the zinc sulphate and lead nitrate showed a decided amount of toxicity, but the Anodons still contained many bacilli at the end of forty-eight hours.

The lead nitrate proved itself to be more toxic than the zinc sulphate solution.

To briefly sum up :—

Of all the different salts experimented with, it would seem that only those of iron are likely to be of use in the purification of suspected infected shell-fish.

Salts of all the other metals used, either acted detrimentally on the Anodons themselves or were not able to free them from the bacilli in a reasonable time.

The conclusions to be drawn from Part II of this paper may be tabulated as follows :—

(1) A solution of colloidal copper made by passing an electric current through distilled water, using copper electrodes so placed as to give an electric arc in the water, is exceedingly toxic to typhoid bacilli, and even when diluted 100 times will kill them in less than two hours at ordinary temperature.

(2) Salts of copper have a harmful effect on Anodons, and the stronger solutions rapidly kill them, whilst the weaker solutions are not able to purify Anodons infected with typhoid bacilli.

It is, therefore, impracticable to attempt to use salts of copper in the purification of shell-fish.

(3) Ferrous sulphate and ferric chloride solutions exert a beneficial action on Anodons placed in them, and the 1 in 1,000 solutions are able to practically free infected Anodons from typhoid bacilli in twenty-five hours.

(4) It is probable that a practicable method of purifying oysters contaminated with typhoid may be discovered by employing some salt of iron for the purpose.

(5) Salts of silver are very harmful to Anodons, and even 1 in 10,000 of M silver nitrate will kill them in less than a week.

(6) Sulphate of zinc and lead nitrate in the stronger solutions are harmful to Anodons, and in weaker solutions, although they exert a decidedly toxic action on typhoid bacilli, are unable to entirely free infected Anodons.

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NOTE ON THE CHEMICAL COMPOSITION AND PHYSICAL PROPERTIES OF RENAL CALCULI

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A series of twenty-two calculi in all was investigated, derived from a fairly wide distribution in South-west Lancashire and North Wales, the object of the study being chiefly to determine whether there was anything in the appearance of a calculus which would give a fairly reliable clue to its chemical composition.

The results are concisely recorded in the subjoined table, which shows that analysis alone can determine the nature of a calculus, the pigmentation, surface, and hardness which are usually relied upon for the classification of calculi being exceedingly untrustworthy.

An important result obtained as far as this series at least is concerned is that uric acid either free or as urates is an exceedingly rare constituent, for uric acid or urates were completely absent in nineteen out of the twenty-two stones, and only traces were found in the three cases where a faint positive result was obtained with the murexide test.

Of equal importance is the unexpected fact that oxalic acid, combined usually with calcium, was found in every one of the stones examined, although the previous literature places this as a very much rarer constituent than uric acid. Phosphates were also commonly present, although this is usually described as only an occasional constituent.

In two of the stones only were carbonates found, but in these two they were present in considerable quantity. These two stones also contained oxalates and phosphates.

In regard to the physical property of hardness, on which great

stress has been laid by earlier observers, in relationship to chemical composition, I have found as far as the simple test of crushing goes that the hardness is very little, if any, related to the chemical composition, and would suggest that the rate and process of formation of the stone is of much more importance, the compactly laid down glossy stone, probably of slower growth, being most difficult to crush, whatever its chemical composition.

In the previous literature—in addition to rarer forms of calculi which need not here be discussed, since none of them were found in the series—there were three chief forms of calculi recognised : (*a*) the pure uric acid calculus, usually described as of a yellowish or brownish colour, rough and crystalline on the surface, and brittle ; (*b*) the urate calculus, containing urates chiefly of magnesium and ammonium along with excess of uric acid, and oxalate of calcium and ammonium magnesium phosphate (these stones were supposed to grow larger than the pure uric acid stones, and were described as having a smooth surface and pale colour) ; (*c*) the oxalate calculus, which was described as having a brown colour and a spinous surface full of irregular projections of a rounded or club-like form (these are said to be much less frequent in occurrence).

The present series of analyses furnishes no experimental evidence to support this classification. As above stated, oxalates are the constituent *par excellence* in this series, being present in every one of the twenty-two calculi examined, and in the majority of the cases in preponderating amount above all the other constituents present. Further, the smoothness and pigmentation give no information as to chemical constitution in the series here examined, and these appear to me to depend much more on the rate of deposit and the natural amount of pigment present in the urine during the period that the stone is being deposited.

It would have been quite impossible for anyone not knowing the three stones in the series which contained uric acid to pick these out from the remainder from their physical appearance, and their pigmentation was very like that of the rest of the series, in which, as above stated, uric acid was entirely absent.

In conducting the analyses, the excellent table of Haller was followed as a routine. Since the amount of oxalates in nearly all the calculi examined was preponderating, it was thought that this might mask the presence of *small* amounts of uric acid, and in order to test this point the following experiment was carried out with an artificial mixture.

A solution of calcium chloride was added to one of oxalic acid, and the insoluble calcium oxalate was separated off. To this precipitated calcium oxalate the smallest trace of uric acid was then added, the proportion of uric acid in the mixture being not more than 1 in 1,000. This mixture was placed in a porcelain dish, a drop of nitric acid added as in the murexide test, and the whole evaporated to dryness.

The residue was quite white, and did not show the usual reddish brown residue of appreciable amounts of uric acid, so that the small trace of uric acid obviously was here concealed by the excess of oxalates.

However, on the addition of ammonia even this trace showed up positively. For a pink coloration could now be detected; this turned purple on the addition of caustic soda. The murexide test was hence here quite positive, and shown to be a very delicate test.

The delicacy of this control assured me that no appreciable amount of uric acid was present save in the three cases in which positive results were obtained.

Table of Analyses with Short Description of Each Calculus

Initials	Percentage of Moisture	Percentage of Incombustible Material	Percentage of Combustible Material	Carbonates	Oxalates	Phosphates	Uric Acid
I.—Mrs. G. Brownish colour, spinous projections	6	43·6	56·4	Absent	Present	Present	Trace
II.—M. L. Shape-cast of kidney pelvis, chocolate colour, with whitish crystals on surface	2·89	26·7	73·3	Absent	Present	Present	Present
III.—E. B. Brownish, glossy, smooth	1·92	43·86	56·14	Present	Present	Absent	Absent
IV.—W. H. H. Greyish, spinous projections	9·7	62·2	37·8	Absent	Present	Present	Absent

TABLE OF ANALYSES WITH SHORT DESCRIPTION OF EACH CALCULUS—*Continued*

Initials	Percentage of Moisture	Percentage of Incombustible Material	Percentage of Combustible Material	Carbonates	Oxalates	Phosphates	Uric Acid
V.—A. E. Oval, greyish rind, cut surface brownish and glossy	3·3	34·5	65·5	Absent	Present	Present	Absent
VI.—F. H. Greyish, brittle	4·13	71·91	28·09	Absent	Present	Present	Absent
VII.—E. K. Shaped to renal pelvis, brittle, greyish-brown	3·8	72	28	Absent	Present	Present	Absent
VIII.—W. G. B. Greyish and spinous	2·09	55·94	41·16	Absent	Present	Present	Absent
IX.—W. E. P. Light brown and spinous	12·2	28·1	71·9	Absent	Present	Present	Absent
X.—Mrs. T. Brownish, irregular, brittle	2·32	59·07	40·93	Absent	Present	Present	Absent
XI.—P J. H. Greyish and spinous	2·15	59·14	40·86	Absent	Present	Present	Absent
XII.—E W. Whitish, irregular surface	5·92	75·6	24·4	Trace	Trace	Large quantity	Absent
XIII.—H. C. Chocolate colour, projections correspond to calyces, glossy	2·3	44·5	54·45	Absent	Present	Present	Present
XIV.—G. W. Large, whitish, brittle	7·6	65·5	34·5	Absent	Present	Present	Absent
XV.—J. D. Oval, greyish, spinous, brittle	5·4	62·8	37·2	Absent	Present	Present	Absent
XVI.—H. H. S. Oval, whitish, and smooth	4·7	76·2	23·8	Absent	Present	Present	Absent
XVII.—M. C. Greyish, irregular, brittle	5·5	66·9	33·1	Absent	Present	Present	Absent

Table of Analyses with Short Description of Each Calculus—*Continued*

Initials	Percentage of Moisture	Percentage of Incombustible Material	Percentage of Combustible Material	Carbonates	Oxalates	Phosphates	Uric Acid
XVIII.—Dr. H. Brownish and spinous	5·67	47·73	52·27	Present	Present	Present	Absent
XIX.—A. L. Surface chocolate and smooth	2·02	40·6	59·4	Absent	Present	Present	Absent
XX—Nurse D. Rind light brown and spinous, cut surface chocolate	3·07	38·5	61·5	Absent	Present	Present	Absent
XXI.—E. L. Oval, chocolate colour, glossy	2·27	43·3	56·7	Absent	Present	Present	Absent
XXII.—Mrs. W. Rind chocolate, cut surface greyish	1·6	32·8	67	Absent	Present	Present	Absent

Note.—Regarding other constituents present calcium and sodium were always found in every calculus of the series. Xanthin cystin and other rare constituents were usually tested for, but were invariably found absent.

The twenty-two samples examined were all from cases occurring in the practice of Mr. W. Thelwall Thomas, F.R.C.S., to whom my thanks are due for the suggestion of this work, as also for his kindness in placing the material at my disposal. My thanks are also due to Professor B. Moore for allowing me to work in his department, and for his help at all times.

Conclusions

1. The commonest constituent by far in this series of calculi is oxalate, present chiefly as the calcium salt.

2. Uric acid in any form was found to be extremely rare, and absent in nineteen out of twenty-two cases.

3. Neither pigmentation, hardness nor surface of the stone are any criterion of its chemical composition, and depend in all probability more upon the physical and physiological conditions while the stone is being deposited.

ON THE PRESENCE OF AN OXIDISING-ENZYME IN THE LATEX OF *HEVEA BRASILIENSIS*

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The following note is intended rather as an addition to a recent paper published in this Journal on the presence of oxydases in india-rubber than as an independent communication on this subject. The question of oxidising-enzymes in indiarubber and in the latex secretion from which this is derived has been fully dealt with in my original paper,[1] and will not, therefore, be discussed again here. As this subject appears to me, however, to be an important one, both biologically in regard to the function of the caoutchouc in the latex of the plant, and commercially in view of possible improvements in the preparation of raw rubber, and furthermore as only indirect evidence was available at the time for the presence of an oxydase in the raw Para rubber examined, it may not be out of place to record here that since the previous communication was written I have had the opportunity of examining four separate samples of latex from *Hevea brasiliensis* (Para rubber), and have found in each marked evidence of the presence of an oxidising-enzyme.

The latices investigated were collected on the Jugra Estate, Ceylon,[2] and were preserved according to my directions by methods which will be described in their proper place. The samples arrived here in good condition, and showed no signs of coagulation macroscopically or microscopically.

For the examination of the latices for oxidising-enzymes the same methods were employed as were described in connection with *Funtumia elastica*.[3] The latex was dialysed for twenty-four hours

1. *Bio-Chemical Journal*, Vol. III, No. 4, p. 165, 1908.

2. I should like to take this opportunity of expressing my thanks to Messrs. Edward Lawrence & Co., of Liverpool, for the care which they have taken in collecting, and for their kindness in providing me with this material.

3. *Loc. cit.*, p. 175.

into running water in order to remove products which might interfere with the reactions. The dialysed latex was then tested with the reagent for detecting the presence of oxidising-enzymes, both with and without hydrogen peroxide. Controls in this case consisted of latex which had been made very faintly alkaline and carefully raised to 80° C., and kept at this temperature for five minutes, then cooled and neutralised immediately before use. In this connection it may be well to point out that only the alkaline or neutral reagents are suitable for the direct determination of oxidising-enzymes in the latex, for those reagents which react best in a faintly acid medium or in alcoholic solution (tincture of guaiacum) produce rapid coagulation of the latex, and give uncertain results.

Each of the four samples of Hevea latex examined was found to give a marked positive reaction without hydrogen peroxide, and a still more intense reaction in presence of this chemical when the indophenol mixture of Röhmann and Spitzer, the hydrochinon and the *p*-phenylene-diamine reagents, were employed as indicators.

Furthermore, by diluting the latices with water and then coagulating with 40 per cent. alcohol, it was found possible by addition of absolute alcohol to the watery mother-liquor to separate the oxidase in an impure state in the form of a gummy mass, which, on drying in vacuo, left a small quantity of a brown vitreous solid. This solid when dissolved in water gave all the reactions of a powerful oxydase.

The chemical properties of this oxidising-enzyme in the latex of *Hevea brasiliensis* have not yet been studied, but the present note serves at least to show that in spite of the negative results of Schidrowitz and Kaye[1] the latex of *Hevea brasiliensis* does actually contain an oxydase enzyme, so that the chain of experimental evidence for the existence of such an enzyme in the so-called insoluble constituent of Para rubber is now complete.

1. *India Rubber Journal*, Vol. XXXIV, No. 1, p. 24 (1907).

EXPERIMENTAL EVIDENCE OF THE LOCAL PRODUCTION OF 'OPSONINS'

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These experiments were originally undertaken in the hope that we might, by the firm application of an elastic bandage and the subsequent inoculation of a vaccine, be able to demonstrate the local production of specific 'opsonins.' Before proceeding to inoculate we felt that it was necessary to test the local and general effects upon the opsonic index produced by the bandage alone. The alterations in the index were so marked that our original purpose was frustrated, as it would have been impossible to say to what extent variations obtained were due to the vaccine.

We are, therefore, left with a series of experiments demonstrating the local and general effects upon the opsonic index resulting from the application to a normal individual of a Bier's bandage. The experiments may be divided into three groups.

A. Evidence that the index of the subject was normal to the Diplococcus Intracellularis Meningitidis (Weichselbaum).[1]

B. Local and general effects upon the index when an elastic bandage was applied with extreme severity.

C. Local and general effects upon the index when an elastic bandage was applied with less severity.

In all the experiments the emulsion was made from a twenty-four hours' old 'nasgar'[2] culture. Leucocytes (2 vols.), emulsion (1 vol.),

1. We selected the Meningococcus because we had already worked with it continuously for nine months in the treatment of patients, and were also so engaged at the time of commencing the experiments.

2 A. Ascitic fluid, 15 c.c.; distilled water, 35 c.c.; nutrose, 1 gramme. B. Ordinary peptone agar. Mix one part of A with two parts of B, steam thirty minutes, filter, place in tubes. Sterilise. (Gordon)

and serum (1 vol.) were always freshly prepared (not more than four hours old), and were incubated for ten minutes at 37° C. One hundred leucocytes were counted. In the majority of the experiments the counter did not know until he had finished to which persons the various films referred, as the slides were mixed with those of patients under treatment. As a rule, he did not know his figures at all, as they were taken down for him by another person. The slides were stained by the following method :—Twenty drops of Leishman's stain for thirty-five seconds ; ten drops of distilled water added, carefully mixed and left for six minutes ; washed in three changes of distilled water for twenty seconds altogether, and dried high up over a bunsen flame.

A.—Proof that the Index of the Subject was Normal to the Meningococcus

Six preliminary observations were made, four on successive days. (See No. 1 on chart.) The culture employed on the first four occasions had been derived from a case seven days previously ; that on the last two had been isolated 138 days before the first of these experiments. A different control blood was used on each occasion. Twice there was only a difference of one coccus between the subject and the control, viz., 135-136 and 229-228. The subject had, then, an index normal (0·9-1·1) to two strains of meningococcus of markedly different ages. The subsequent experiments confirmed this, as the index was always taken before the application of the bandage, and invariably fell within these limits.

B.—Local and General Effects upon the Index when an Elastic Bandage was Applied with Extreme Severity

(See Nos. 2, 3 and 4 on chart)

Five yards of Esmarch's plain rubber bandage were applied to the left forearm just below the elbow for an hour. Severe pain was induced in the hand during the experiment, and tingling and numbness for several hours afterwards. The subject was covered with cold perspiration, and was somewhat collapsed. The limb below

the bandage became intensely cyanosed at first, but towards the end of the hour was leaden-coloured. The pulse was absent at the wrist, and sufficient blood for the index was only obtained by pricking deeply the pulps of all the fingers.

Results.—In each case there was a very distinct rise in the index of the left hand at the end of the hour (1·7, 1·5, 1·9). In one case (Experiment No. 4) this rise had already begun at the end of half an hour. In this experiment indices were taken from both hands, and showed that the rise was confined to the left. Experiments Nos. 2 and 4 demonstrate that twenty-four hours later the index had returned to normal.

C.—Local and General Effects upon the Index when the Bandage was Applied with Less Severity

(See Nos. 5, 6, 7, 8, and 9 on chart)

In these experiments the bandage was applied with sufficient firmness to produce apparently the same amount of stasis and discoloration, but relatively little pain. That the constriction was sufficient to isolate the limb from the general circulation is seen in the chart, which shows that variations in the index, where they occurred at all, were confined to the left hand.

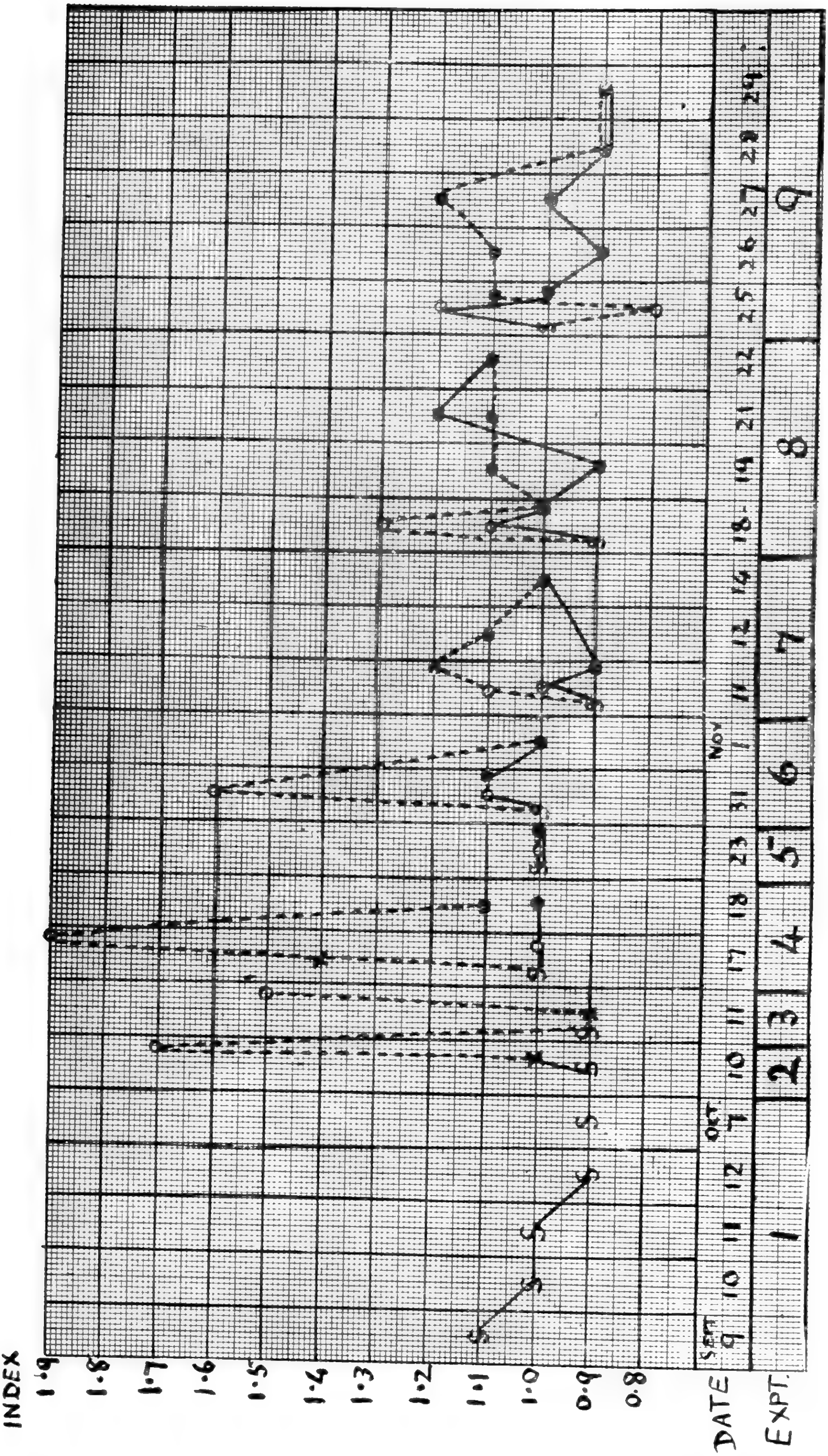
Experiments Nos. 5 and 9 show no result. In No. 5 the index readings are worth reporting in detail, as showing the consistency of the results. Control: 77 cocci in 100 leucocytes. Four readings from the subject (both hands) at the various stages of the experiment: 79, 76, 74, and 78 cocci in 100 leucocytes. In No. 9 a rise to 1·2 on the second day can hardly have been due to the application of the bandage.

If we consider Experiments Nos. 5, 6, 7, 8, and 9 together, we find that under the conditions of the observations the rise in the index is never more than moderate, and may be absent. In none of the experiments does the effect last more than twenty-four hours.

Conclusions

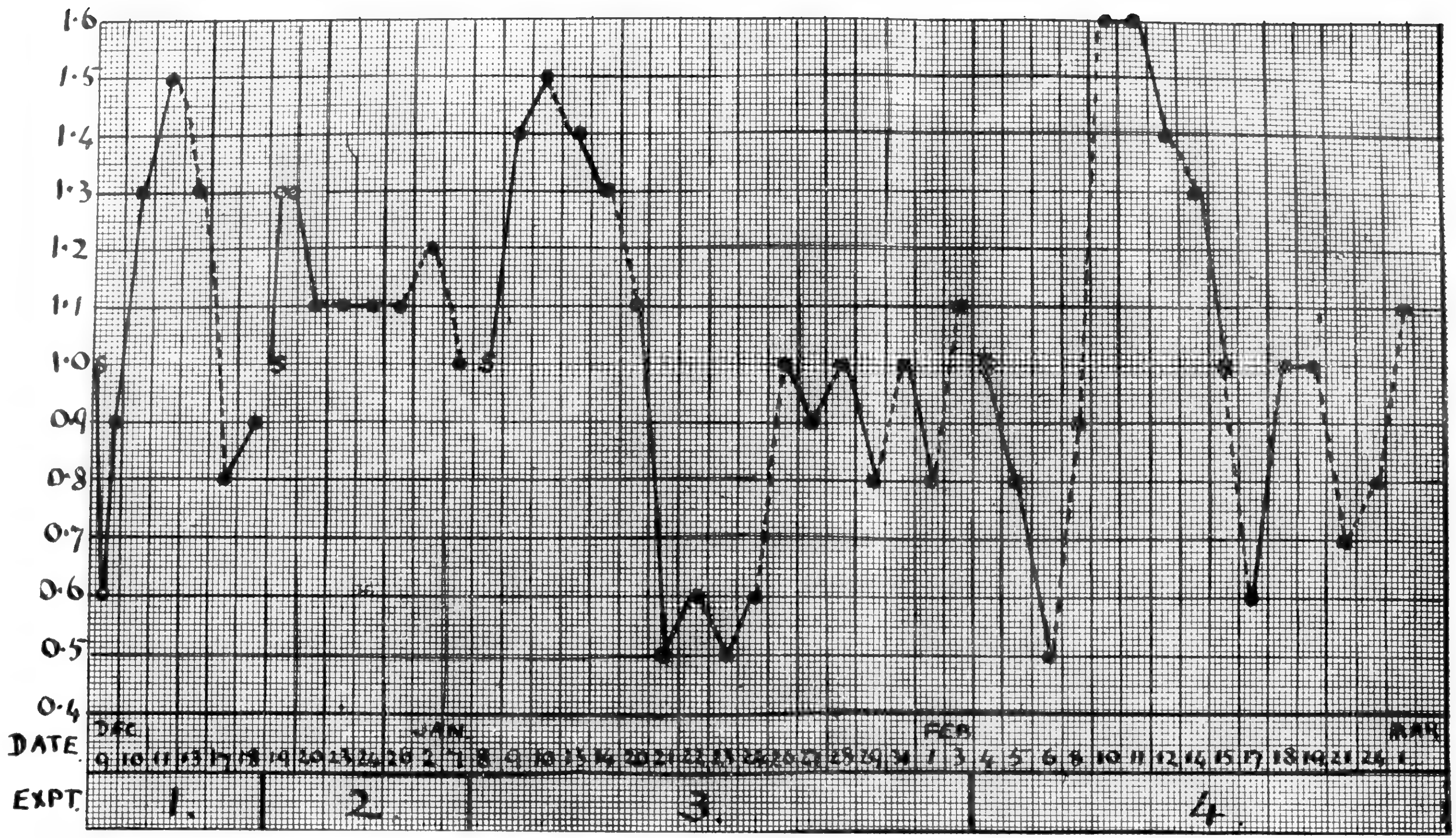
It would appear that to produce a marked rise in the index a Bier's bandage when applied for one hour must be sufficiently firm to cause very considerable pain. This leaves untouched the question of a more moderate application extending over a longer period of time; but, from a consideration of the above experiments and the variations in the rises obtained, it may be doubted whether an application however long which does not isolate the limb will have any appreciable effect on the index. It is certain that the results will be irregular in character. The irregularity of the indices seems to us to be of interest, if only from the fact that it confirms the clinical experience of those who use Bier's bandage as a therapeutic measure, and find that the beneficial effects vary greatly.

A further point is brought out, namely, that the alteration in amount of the ' opsonins ' (or such part of them as can be gauged by the index) was purely local, and that, therefore, no other tissues of the body were required for its manifestation than those present in the bandaged forearm.



OPSONIC INDEX CHART

S Index before experiment.
- - - Index curve of left hand.
—— Index curve of right hand.
X Index of left hand half an hour after application of bandage.
O Index of either hand at time of removal of bandage.
● Index of either hand one hour after removal of bandage and on succeeding days.



OPSONIC INDEX CHART

S Index before experiment. O Index one hour later. ● Index two hours later and on subsequent occasions.

—— joins indices with an interval of twenty-four hours or less. - - - - joins indices with a longer interval.

EXPERIMENTAL INOCULATION OF MENINGO-COCCIC VACCINE

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From the Department of Hygiene, University of Liverpool

(*Received July 2nd,* 1908)

While treating cases of epidemic cerebro-spinal meningitis by the vaccine method we were struck by the difficulties experienced in estimating the dosage. The following investigation, therefore, was undertaken to determine the limit of dosage for a normal adult, and in the hope that at the same time light would be shed on the degree of immunity produced.

Three points seem to be established by the experiments :—(1) That a dose of 222 million organisms was as much as the subject could bear with safety. (2) That the inoculation of a vaccine is not likely to be of much value as a prophylactic. (3) That a vaccine three weeks old has not lost its potency.

Technique. A. *Method of Preparing and Staining Slides for the Index.*—Two volumes of corpuscles were mixed with one volume of emulsion and one volume of serum, incubated at 37° C. for ten minutes, and films stained in the following way :—Leishman's stain twenty drops for thirty-five seconds ; distilled water ten drops added, carefully mixed and left for six minutes ; film washed in three changes of distilled water for twenty seconds altogether, and dried high over a bunsen flame. The emulsion was on each occasion prepared from a twenty-four hours' sub-culture on 'nasgar'[1] of a strain 201 days old on the date of the first inoculation (December 9th, 1907).

B. *Method of Counting.*—The preparation of the films was always done by one person, and the counting also by one person ;

1. For composition, see previous paper.

but before the counting was begun the slides were re-labelled by someone not concerned, and mixed with the slides of patients under treatment at the time. Every effort, therefore, was made to reduce any possible mental bias. One hundred leucocytes were counted on every occasion.

C. *Preparation of the Vaccine.*—The vaccine was prepared from twenty-four hours old sub-cultures on 'nasgar' of the same strain as that employed for the index. Normal saline was the diluting fluid, and after standardisation sterilisation was effected by heating in a water bath to 60° C. for thirty minutes. Sterility was confirmed by incubating for twenty-four hours on 'nasgar.' Until used, phials of vaccine were preserved in a cool dark place.

D. *Method of Standardising Vaccine.*—All the vaccines prepared in this laboratory are now standardised by this method.[1] A Thoma-Zeiss cell is employed, covered in by a thin slip chosen in the following way :—A series of ordinary thin (No. 1) 7/8 inch slips are carefully cleaned, dried, and applied lightly to the plate glass slip provided with the apparatus, after gently breathing upon the surfaces to be opposed. The least pressure that will cause them to adhere is used, and the slip chosen which shows when held up to the light the best distribution of Newton's rings. Only a few slips from each ounce are satisfactory in this respect. The slips so selected may, of course, be used many times over. The diluting fluid is sodium chloride 0·1 per cent., Giemsa's stain 5 per cent., in distilled water ; filtered or preferably allowed to stand for a few days. Before use 5 per cent. of formalin is added. The organisms gravitate to the bottom, the formalin inhibiting movement, and the trace of salt assisting the staining. We are indebted to Major Harrison for the suggestion that formalin should be added. Either the red or the white corpuscle pipette may be used, according to the dilution required. A small plug of cotton wool is placed in the end, and the pipette is sterilised in the hot-air steriliser. The usual precautions as to the size and placing of the drop are observed, and the 1/12 oil immersion

1. The method was described by one of us at a Pathological Meeting of the Liverpool Medical Institution, on March 5th, 1908, and a short description appeared in the *Lancet* of March 14th, 1908, in its report of that meeting.

lens is applied after dropping excess of oil on the slip. It is advisable to first of all bring the scale into good position with the 1/6 lens. Counting, with the condenser somewhat lowered, may be commenced within an hour; and as a rule it is quite sufficient to examine three sets of sixteen small squares. It is well to focus through the *depth* of the cell, as an occasional organism may be found floating, but the vast majority lie placidly at the bottom and may be rapidly counted.

The method has been controlled by using for the same vaccine different dilutions in two similar pipettes, and by using simultaneously both the red and the white corpuscle pipette. That the error from irregularity of the coverslip or possible cupping from surface tension is negligible can be judged from the even distribution of the organisms in the small squares. Using a red corpuscle pipette and taking vaccine up to the second cross-marking from the point (*i.e.*, a dilution of 1 : 500), the average number of organisms per small square is five in a vaccine containing ten millions per cubic millimetre. By using the fullest dilution with the red corpuscle pipette it would be possible to standardise a vaccine containing 100 millions per cubic millimetre.

Control Experiments.—The index of the subject of the experiments was tested eight times against five different sera before commencing the inoculations, and five times against four different sera afterwards. On every occasion it was found to be normal—that is to say, between 0·9 and 1·1. Although we accept this range as a basis, in actual fact our results so closely approximate to unity that we feel it is advisable to give them for these thirteen observations—especially so in view of the criticisms that have been persistently levelled at the accuracy of the index :—193-214, 135-136, 229-228, 202-219, 62-59, 30-32, 70-72, 76-78, 80-81, 76-74, 98-97, 71-77, 77-80.

Experiment No. 1.—9th December, 1907. Four cubic millimetres (containing 32 millions) of a vaccine prepared four days previously were injected into the left forearm. At the end of one hour an index from each hand showed a very marked drop, which was recovered from one hour later. Till 13th December there was a well-marked positive phase, with a maximum of 1·5 (each hand) on 11th December. A second sub-normal phase was present at the next examination (17th December). One day later

the index was normal (normal also to staphylococcus aureus), and remained so on the following day, when the next injection was given.

Reaction.—There was no general reaction. Local swelling appeared one hour after inoculation, increased steadily during the next few hours to the size of a pigeon's egg. There was not much redness. Twenty-four hours later the swelling was still marked, but flatter and more diffuse ; the skin for about four square inches was hard and tender to pressure, but otherwise painless ; the edges were distinctly raised, and the colour bright red. On 11th December the redness around the puncture was less, but its area had increased by one-half inch all round ; less hardness was present, except immediately around the puncture, where there was still a little swelling. On 12th December there was merely a little thickening and bronzing round the site of inoculation.

Experiment No. 2.—19th December, 1907. Eight cubic millimetres (containing 68 millions) of a vaccine prepared fourteen days previously were injected subcutaneously into the left forearm. One hour later the index of each hand had risen (1·3 L., 1·2 R.). Readings till 26th December were within the normal range, but at its upper limit ; on the 2nd January, 1908, 1·2 ; and on the 7th and 8th January, normal. The index on the 23rd December was 1·1 to two strains of the meningococcus (215 and 112 days old respectively).

Reaction.—There was no general reaction. Local swelling about one inch in diameter appeared one hour after the inoculation ; twenty-four hours later there was swelling and uniform redness for six inches around the puncture ; degree and course very like the first inoculation.

Experiment No. 3.—8th January, 1908. Twenty cubic millimetres (containing 100 millions) prepared two days previously were injected subcutaneously into the left forearm. Twenty-four hours later the index had risen to 1·4, and it remained above normal on the 10th, 13th, and 14th (maximum 1·5 on the 10th). It was normal on the 20th, and then rapidly fell to 0·5, in which region it remained for four days. It then rose to normal, within a range of 0·2 of which it oscillated until the next inoculation on February 4th. During the second sub-normal phase indices taken against the staphylococcus albus and citreus were found to be normal.

Reaction.—There was some headache and malaise for twenty-four hours. On the day after the injection there had developed round the puncture a tender but otherwise painless area of brawny redness four by four and a half inches in extent. The axillary glands were unaffected. On the 10th the area of redness was larger, but the colour less intense ; on the 11th the area of redness was only two inches in extent around the puncture. By the 21st January the area around the puncture had gone through stages very similar to calf lymph vaccination ; on this day the scabs were removed. Local tenderness had ceased.

Experiment No. 4.—4th February, 1908. Thirty cubic millimetres (containing 222 millions) of a vaccine prepared eighteen days previously were injected subcutaneously into the left forearm. A negative phase appeared on the 5th, and was pronounced on

the 6th. On the 8th (ninety-six hours after the injection) the index was once more within the normal range. The indices on the 10th, 11th, 12th, and 14th showed a well-marked rise (highest 1·6 on the 10th and 11th).

To this again succeeded an oscillating but mainly sub-normal phase lasting at least a week. As confirmation that this oscillation was actually present, it is of interest to note that the blood of a patient under treatment at the same time gave, with a control common to both, a very even curve.

Reaction.—The injection was given at 5.30 p.m.; the subject had felt quite well all afternoon. At 6.15 p.m. there was a feeling of nausea accompanied by severe vertical headache and rigor. At 7.30 p.m. vomiting occurred, and some diarrhoea; the temperature was 103 F., and the pulse rapid; headache was very severe, and a restless night was spent. In the morning the temperature had fallen to normal, and the subject felt better except for the headache, which persisted for two days. There was considerable pain behind the eyes for thirty-six hours, and pain, but no stiffness, at the back of the neck for two days; this persisted to a slight extent for six days. There was no pain in the back. The skin over the head and neck was tender. On the second day after the inoculation severe herpes appeared on the right side of the lower lip and left ear, which were greatly swollen and covered with vesicles. Locally there was a tender area five inches in extent; redness appeared around the puncture very similar in appearance and course to the previous experiments. No local vesiculation occurred; the axillary glands were unaffected.

Summary

These experiments form a series, with a longest interval during which no indices were taken of seven days. Succeeding inoculations were only given when the index change from the preceding inoculation had returned to normal.

Primary Negative Phase.—Apart from a drop in Experiment No. 1, lasting less than two hours but confirmed in each hand, there was no initial negative phase after the first three inoculations, but a very marked one (four days) after the fourth.

Positive Phase.—All four inoculations produced a positive phase, lasting in No. 1 not longer than a week—certainly four days.

No. 2 (? fourteen days), since a series of four consecutive indices at the upper limit of the normal range *may* be considered a rise, especially when preceded and followed by an undoubted rise, the former confirmed in each hand.

No. 3, not longer than twelve days—certainly six days.

No. 4, six days.

In every case the highest point was reached within two days of the development of the positive phase.

Second Negative Phase.—This followed the positive phase in three cases (Nos. 1, 3, and 4), lasting one to two days, four to five days, and a week or more respectively. With the larger doses this sub-normal phase showed a very distinct and somewhat prolonged oscillation, with, however, a sub-normal average—that is to say, the original positive and negative phases were merely the first in a series of which the amplitude steadily diminished. The number of days after each injection before the index had settled at normal worked out in—

No. 1—Ten days.
,, 2—Nineteen days.
,, 3—About twenty-four days.
,, 4—About twenty-six days.

Reaction.—The reaction, local and general, increased *pari passu* with the dosage of vaccine, and irrespective of its age, up to three weeks.

Agglutination.—This was tested on the 6th and 9th of December, and the 15th February, by the loop method, at laboratory temperature, 1/6 lens, time four hours. In dilutions of 1-10, 1-20, 1-40, the results were negative. Apparently the extent of saturation required to produce agglutination was much greater than our experiments reached.

Conclusions

Limit of Dose.—There can be little doubt that in this particular subject a dose of 222 millions even after three preceding inoculations was markedly excessive, leading to prolonged sub-normal phases, with a positive phase no longer than in the preceding experiment. Even the dose of 100 millions produced a second sub-normal phase to be avoided. It would seem, therefore, that the maximum useful dose for an adult is well under 100 millions; as by the smaller doses

the rise obtained was as good, and the sub-normal phases were less marked.

Immunity Produced.—It appears that the immunising power of a meningococcus vaccine so made and administered is not of much practical service as a prophylactic, and might, indeed, do harm from the production of supersensitisation. That the immunity conferred by an attack of the disease in a human subject is not of very long duration is shown by a case which we had under vaccine treatment in July, 1907, in collaboration with Dr. Rundle and Dr. Williams, of the Liverpool City Hospital, Fazakerley. During the illness a dose of 21 millions produced no local reaction. In November, 1907, the patient came to see us; the index then was 1·4 (tested once). During December, January, and February it fell to normal, and as the patient was complaining of variable but apparently increasing deafness a dose of 30 millions was injected subcutaneously.

The result obtained was in all respects comparable to a similar dose in the subject of these experiments. That is to say, six months after her illness the patient had returned to the normal state as tested by the local reaction after the inoculation of a moderate dose of vaccine. The patient's serum agglutinated her own organism once during the illness in a dilution of 1 : 25 at laboratory temperature, but on no occasion after recovery.

CHANGES IN THE CHEMICAL COMPOSITION OF THE HERRING DURING THE REPRODUCTIVE PERIOD

By T. H. MILROY, *Professor of Physiology, Queen's College, Belfast.*

(*Received July 7th*, 1908).

The chemical changes which occur in the salmon during the various periods of its life history have been worked out with great care and thoroughness by Miescher,[1] Noël Paton[2] and others, and the results which have been obtained have greatly increased our knowledge of the relationship between the reproductive organs and the muscles of this fish.

It is natural to suppose that a somewhat similar cycle of changes might occur in the herring, and as this fish constitutes such an important article of diet, it was deemed advisable to study the changes in its composition at various seasons of the year.

At the request of the Scottish Fishery Board I examined fish obtained at various seasons and in different stages of reproductive activity. In the first place I examined fish caught on the West Coast of Scotland (Loch Fyne District) for a period of one year, starting with fish with immature ovaries or testes, and ending with spent fish.

The fish caught in May showed very immature genitalia, and, as summer and autumn proceeded, the ovaries showed a continuous increase until December, when full maturation was attained.

In January and February the fish were either spawning or spent.

During March and April the fish were spent, but began to show commencing growth of ova in the old ovarian capsules.

A similar investigation was subsequently carried out in the case of East Coast fish, and the results of this examination will be given in a subsequent paper.

The fish in all cases were sent in a double-walled carrier, an ice-salt mixture filling the space between the walls.

1. *Die Histochemischen u. Physiologischen Arbeiten*, by Miescher, 1897.
2. 'The Life History of the Salmon,' *Scottish Fishery Board Reports*, 1898.

The fish were measured and weighed, and the genitalia then removed and also weighed.

The ova were examined microscopically, and their diameter measured with the ocular micrometer.

The length was measured from end of snout to end of tail fin, while the girth was taken in front of the dorsal fin around the thickest part of the fish.

The fish were then skinned, and the muscles passed through a mincing machine. It was found impossible to do more than analyse the muscles and the ovaries. Some analyses were made of different muscles, but the variations in composition were so slight that the plan was abandoned and specimens taken of the general musculature instead.

Methods of Analyses

It was impossible to adopt many precautions which might have been desirable, owing to the necessity for carrying out a large number of analyses rapidly.

The water content of the muscles and ovaries was arrived at by drying the minced substance in vacuo over sulphuric acid.

The total nitrogen was estimated by Kjeldahl's method, and the P_2O_5 by incineration with pure NaOH and KNO_3, and subsequent treatment by the ammonium molybdate method.

The protein percentage in most cases was simply arrived at by converting the total nitrogen into terms of protein, but in the case of the East Coast fish previously mentioned the coagulable protein was estimated by the anhydrous sodium sulphate method.

The fat was estimated in the powdered dry material after thorough admixture with pure silver sand, the powder being in the first place extracted with hot alcohol, and then with ether by Soxhlet's method.

It was thought advisable not only to give the percentage amounts of protein, fat, and P_2O_5, but also the absolute amounts in the collective muscles and genitalia respectively of the same fish.

Tables are also given showing the ratios between the weight of the fish and the reproductive organs at different periods, and also those existing between the more important constituents of the muscles and genitalia.

Analyses of herrings[1] have been made by different investigators, but so far as my knowledge goes no systematic examination of the fish has been made at the different periods of its reproductive life.

Atwater gives the maximal, minimal, and average amounts in the herrings examined by him, but makes no statement as to the condition of the genitalia.

His numbers are the following :—

	Water	Water-free substance	Protein *i.e.*, N × 6·25	Fats	Ash
Maxima	76·11	30·97	19·12	11·01	1·9
Minima	69·03	23·89	15·31	4·89	1·5
Average	72·19	27·90	17·75	8·02	1·69

Results of Analyses of West Coast Herrings

Those fish which were sent for examination during May were smaller than those received at later periods.

The following table gives the necessary information with regard to their condition :—

Table I.—Loch Fyne Herring, May 10th, 1906

Length cm.	Girth cm.	Weight cm.	Condition
25	11½	113	Very immature, previously spent herrings.
23½	11	101	
22½	12	104	There were large numbers of very small ova present, varying in size from 0·07 to 0·15 mm. in diameter
23½	11	102	
23	10½	93	
23	10	90	
23	10½	88	
21½	10½	80	
22½	10½	85	
22	10	80	
21½	10	74	
22	10	75	
Average 22·9 cm.	10·7 cm.	90·4 gm.	

Weight of genitals (fresh) of 12 herrings, 2·5 grammes. The water percentage of these genitals was 71·52.

1. Payen, *Substances Alimentaires*, p. 488; König, *Nahrungsmittel*, Bd. I, pp. 201-7; and Atwater, *U.S. Commissioner's Report on Fish and Fisheries*, 1888, pt. XVI, 1892.

Analyses of the muscles of these herring :—

Female—

(*a*) Amounts stated in percentages of fresh material.

Water	Protein	Fat	P_2O_5
72·69	18·98	7·25	0·68

(*b*) Grammes in the total muscles of the average fish of this series.

Protein	Fat	P_2O_5
11·38	4·35	0·40

These herrings had probably spawned some time between February and April on the Ayrshire coast (Ballantrae spawning beds), and had then passed up Loch Fyne. They were caught near the opening of that Loch. Judging from the amount of fat present, they had, however, probably resumed feeding for more than a month, but the collapsed condition of the large ovarian capsules showed that within a comparatively recent time the fish had discharged their ova.

The high water percentage of the muscles is also characteristic of fish that have been recently spawning.

On the following day some herrings were caught in the same neighbourhood, and these showed even more marked signs of recent spawning. (Table II.)

They were on the average smaller fish, with one exception. This one (No. 10) was examined with the rest because it was evidently in the same condition as the others, which were on the average thinner and lighter than those of the previous set.

As will be seen from the table, the water percentage is higher and the fat lower than in the previous set, while the phosphorus percentage is higher than in any other muscles which were examined.

TABLE II.—MAY

	Length cm.	Girth cm.	Weight gm.	Condition
1	20	8·5	58	The ova were very immature. The weight of the 10 pairs of genitals was 2·4 gm. in the fresh condition.
2	20·5	9	60	
3	20·5	9·5	67	
4	20·5	9·5	67	
5	20·5	9	64	
6	21	9	72	
7	21·5	10	75	
8	22·5	9	75	
9	21	9·5	70	
10	25	11	116	
Average	21·3 cm.	9·4 cm.	72·4 gm.	

Muscle.

(*a*) Amounts stated in percentages of fresh material.

Water	Protein	Fat	P_2O_5
73·01	17·55	5·85	0·82

(*b*) Grammes in the total muscles of the average fish in the series.

Protein	Fat	P_2O_5
8·47	2·82	0·39

Fish caught during June and July were practically in the same condition, there being only slight growth of the genitalia; the weight of the fish gradually increased, but the percentage composition of the muscles altered very slightly.

It is scarcely necessary to give the numerous analyses of June fish which were made, because they were practically identical with those mentioned under the first set of the May fish.

An example is here given of fish caught at the end of July. The ovaries were, although still immature, sufficiently large to allow of their analysis.

July.—The water percentage of the muscles is still high, although slightly below that occurring in the May fish.

The protein percentage is practically the same, and the fat slightly higher than in the average May fish.

The ovaries were larger, with the ova in a slightly more mature

condition, averaging about 0·2 mm. in diameter. Analyses were therefore made of the genitalia.

TABLE III.—JULY

	Length cm.	Girth cm.	Weight gm.	Weight of Genitals gm.
	24	12	116	0·35
	22	11·5	96	0·46
	23·5	12	99	0·20
	23	13	120	0·67
	23	11·5	91	0·20
	22·5	12	105	0·74
	23	12	107	0·33
	24	12·5	124	1·18
	21·5	11	82	0·49
	22·5	12	105	0·43
	22·5	11·5	102	0·39
	22	11	92	0·22
	23	12	97	0·15
	24	13	127	0·50
	22·5	12	100	0·44
	25	13	137	0·53
	24	12·5	119	0·81
	22	11	88	0·20
	22	11	92	0·20
	23	13	120	0·65
Average	22·9	11·9	100·9	0·45

Analyses of the Muscle (fresh) :—

(*a*) Percentages.

Water	Protein	Fat	P_2O_5
71·60	18·18	7·32	0·45

(*b*) Grammes in the total muscles of the average fish.

Protein	Fat	P_2O_5
12·18	4·90	0·30

Analyses of the genitals (fresh) :—

(*a*) Percentages.

Water	Protein	Fat	P_2O_5
72·5	13·52	8·92	0·77

(*b*) Grammes in the amount of genitals present in the average fish of series.

Protein	Fat	P_2O_5
0·05	0·03	0·003

September.—By the middle of September the ovaries were almost 1 gramme in weight, and the ova measured about 0·2 to 0·45 mm. in diameter.

It will be sufficient simply to state the percentage composition of the muscle flesh at this period.

Water	Protein	Fat	P_2O_5
63·68	19·28	11·81	0·64

In all the fish examined about this time the water percentage was at its lowest, and there was also a distinct increase in the amount of fat and protein.

The ovaries at this period had the following percentage composition :—

Water	Protein	Fat	P_2O_5
66·02	18·91	7·34	1·23

The total amounts present in the ovaries of the average fish of this series were, of course, very small, namely :—

Protein	Fat	P_2O_5
0·17	0·07	0·011

The relatively high fat percentage of the ovaries indicates that the degenerated ovarian tissue of the spent fish has not yet been entirely used up.

October.—During this month fish were obtained in different stages of maturity, some with ovaries weighing from 2 to 3 grammes, others weighing about 9 grammes ; while the ova varied from 0·3 to 0·6 mm. in diameter.

By far the larger number of the fish caught at this period were heavier than those obtained at an earlier season, but the changes in composition were of the same kind in all.

As will be seen from Table IV, the most marked alteration in the muscles is the great increase in the fat, the highest percentage being observed in fish caught at this season.

It will be observed from a study of the later series that as the ovaries begin to increase rapidly in size the fish begins to use up the store of fat which has been accumulating in the muscles during the earlier months.

Two series of fish are given in the following table, one (Series A) including those caught during the earlier part of the month, the other (Series B) towards the close of October. (Table IV.)

Under Series B the results of the examination of male fish caught at this season are also given. As will be seen, they correspond very closely to the females.

TABLE IV.—SERIES A (OCTOBER 7TH)

	Length cm.	Girth cm.	Weight gm.	Condition
	28	15	206	The ovaries from these seven herrings weighed collectively 17 gm., and the average size of ova was 0·28 mm.
	27	13·5	150	
	25	13	147	
	24·5	12·5	130	
	23·5	12	115	
	23·5	12·5	122	
	26	13	148	
Average	25 cm.	13 cm.	144·5 gm.	

SERIES B (OCTOBER 20TH)

	Length cm.	Girth cm.	Weight gm.	Condition
	29·5	16	257	The ovaries from these eight herrings weighed collectively 74 gm., and the average size of the ova was 0·59 mm.
	28·5	15·5	220	
	27·5	13·2	167	
	24·5	12·5	129	
	24	12·2	119	
	25	14·5	220	
	29·5	15·5	229	
	28	14	197	
Average	27 cm.	14 cm.	192 gm.	

Series A.

Muscles.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
69·97	12·78	14·25	0·53

(*b*) In total muscles of average fish.

Protein	Fat	P_2O_5
12·39	13·82	0·51

Ovaries.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
78·21	17·26	2·53	0·71

(*b*) In ovaries of average fish.

Protein	Fat	P_2O_5
0·41	0·33	0·01

Series B.

Muscles.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
70·46	14·84	12·70	0·57

(*b*) Per average fish.

Protein	Fat	P_2O_5
18·99	16·25	0·73

Ovaries.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
68·02	22·45	4·80	0·93

(*b*) Per average fish.

Protein	Fat	P_2O_5
2·08	0·44	0·05

Males—

Muscle.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
68·91	16·18	12·63	0·50

(*b*) Per average fish.

Protein	Fat	P_2O_5
17·91	13·98	0·55

Testes.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
72·13	22·62	2·25	0·86

(*b*) Per average fish.

Protein	Fat	P_2O_5
5·42	0·54	0·20

November.—During November there was a rapid increase in the size of the ovaries, the ova measuring about 0·8 mm. in diameter on the average. The water percentage of the muscles was lower than in the preceding month, and the fat showed the first indication of being used up by the fish from the distinct decrease in its amount, while the protein showed a rise.

It is probably about this period that the winter spawning fish begin to take a smaller quantity of food, and, therefore, they fall back upon their fat store as a source of the necessary energy.

The muscles of the male fish are practically of the same composition as those of the female. (Table V.)

TABLE V.—NOVEMBER FISH

I. Females

	Length cm.	Girth cm.	Weight gm.	Weight of ovaries gm.
	30	16·5	275	35·7
	31	16	270	38·2
	33	17·5	326	51·6
	31	16	255	31·8
	30	16	261	25·6
	31	16	260	18·9
	32	16·5	297	31·6
	32	17	290	35·2
	32	16·5	275	31·4
	30	16·5	276	28·8
	32	16·5	304	32
	29	15	210	20·1
	30	15	204	15·4
	29	14	192	15·8
Average	30·8 cm.	16 cm.	264 gm.	29·5 gm.

The ova were from 0·8 to 1 mm. in diameter.

II. Males

	Length cm.	Girth cm.	Weight gm.	Weight of Testes gm.
	31	17	279	44·5
	31	17	316	52
	31	16	277	40·6
	29	14	215	31·4
	30	16	257	40·9
	33	16	296	37·3
	32	16	287	40·1
	30	17	290	41·1
	31	17	303	46·6
	31	16	252	35·6
	31	15·5	230	26·8
	29	14·5	195	24·4
	30	16	255	44·6
Average	30·7 cm.	16 cm.	265·5 gm.	38·9 gm.

Females—

Muscle.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
66·34	19·87	10·85	0·59

(*b*) In total muscles of average fish.

Protein	Fat	P_2O_5
34·97	19·09	1·03

Ovaries.

(*a*) In percentages of fresh material.

Water	Protein	Fat	P_2O_5
68·04	25·04	2·85	0·91

(*b*) Per average fish.

Protein	Fat	P_2O_5
7·38	0·84	0·26

Males—

Muscle.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
68·22	17·94	10·84	0·46

(*b*) Per average fish.

Protein	Fat	P_2O_5
31·77	19·18	0·81

December.—The fish caught during this month were either ready for spawning or had commenced to spawn.

An example will first be given of fish which although probably about to spawn still show a moderately high percentage of fat, and a low percentage of water in the muscles. They were, in fact, in very good condition. The genitalia were, as will be seen from the table, of the maximum weight, and the ova were mature.

Table VI.—Early December Fish

Females

	Length cm.	Girth cm.	Weight gm.	Weight of ovaries. gm.
	31	17·5	326	63
	30·5	15	268	43
	30·5	16·5	272	30
	31	16·5	330	48
	30·5	16	285	47
	31	17·5	318	51
Average	30·7 cm.	16·5 cm.	299·8 gm.	47 gm.

Males

	Length cm.	Girth cm.	Weight gm.	Weight of testes gm.
	31	17	298	61
	31	17	320	55
Average	31 cm.	17 cm.	309 gm.	58 gm.

Analyses :—

Females—

Muscle.—

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
67·36	20·56	8·18	0·68

(*b*) Per average fish.

Protein	Fat	P_2O_5
41·12	16·36	1·37

Ovaries.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
67·33	25·72	2·89	1·03

(*b*) Per average fish.

Protein	Fat	P_2O_5
12·08	1·35	0·48

Males—

Muscle.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
68·31	21·45	9·24	0·73

(*b*) Per average fish.

Protein	Fat	P_2O_5
44·18	19·03	1·51

Testes.

(*a*) In percentages.

Water	Protein	Fat	$P O_5$
72·10	22·05	3·73	2·10

(*b*) Per average fish.

Protein	Fat	P_2O_5
12·78	2·16	1·21

The following table (VII) gives the results of the examination of herrings obtained farther North. They had commenced spawning ; in fact, had discharged probably about the half of their store of ova. The ova present were, of course, fully ripe. The fish were smaller and in much poorer condition than those of the previous series.

As they are not from the same neighbourhood they are not strictly comparable with the preceding series, but it was impossible to obtain herrings from Loch Fyne during late December and January.

These, however, belong to the same class of herrings as those which are given in the subsequent January and February series.

Two herrings (females) were taken for analysis from a batch containing fish of approximately the same size. Their measurements were :—

TABLE VII.—LATE DECEMBER FISH

	Length cm.	Girth cm.	Weight gm.	Weight of ovaries gm.
	29	14·5	195	26
	29	14·5	194	35
Average	29 cm.	14·5 cm.	194·5 gm.	30·5 gm.

Size of ova, 0·8 to 1·2 mm.

Analyses :—

Muscle.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
72·50	23·01	2·75	0·77

(*b*) Per average fish (total in muscles).

Protein	Fat	P_2O_5
28·76	3·4	0·96

Ovaries.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
65·73	27·76	3·34	1·19

(*b*) Per average fish.

Protein	Fat	P_2O_5
8·46	1·02	0·34

The most striking changes in the muscles of these herrings are the marked fall in the fat percentage and the rise in the water percentage.

This marked decrease in the fat was always found to take place either just at the onset of spawning or after spawning had been in progress for some time.

In the case of the fish from the East Coast of Scotland spawning in early autumn, the fat percentage did not sink so low, and had often reached its minimum in the so-called ‘ full ’ fish—that is, the herring with mature ovaries.

This will be seen from a study of the analyses of these fish which will be given in a later paper.

The fat percentage of the ovaries has risen slightly above that of the mature fish prior to spawning, this being characteristic of the spawning process.

January (Table VIII).—Some of the late December fish showed the same characters as those taken in January.

They could not be obtained at this time in Loch Fyne, but some were analysed which came from farther North. They were in nearly all cases slightly smaller fish. Two series are given, one with spawning proceeding rapidly, the other in the spent condition. The male fish were not examined.

TABLE VIII.—JANUARY. SERIES A (spawning)

	Length cm.	Girth cm.	Weight gm.	Weight of ovaries gm.
	27·5	13·5	165	18
	26·5	14	157	14·8
	26	13·5	141	25
	26	12	127	11·5
	26·5	13	168	20·3
	26	14	160	18·3
	26	13	150	19·4
	25	12	115	10·5
	26	13·5	150	18·8
Average	26 cm.	13 cm.	148 gm.	17·4 gm.

Series A.

Muscles.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
74·12	18·91	2·02	0·66

(*b*) Per average fish.

Protein	Fat	P_2O_5
18·15	1·93	0·61

Ovaries.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
70·00	24·75	3·72	0·93

(*b*) Per average fish.

Protein	Fat	P_2O_5
4·23	0·63	0·16

These fish were in much the same condition as those of the preceding series, but they had a higher water and a lower protein and fat percentage.

Among the fish obtained at this time there were two which were completely spent, the ovaries being collapsed, and only a few remaining ova seen in the collapsed tissue.

TABLE IX.—SERIES B (spent)

	Length cm.	Girth cm.	Weight gm.
	23	12	153
	28	12	140
Average	25·5 cm.	12 cm.	146·5 gm.

Series B.

Muscles.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
75·30	19·69	1·55	0·77

(*b*) Per average fish.

Protein	Fat	P_2O_5
18·53	1·51	0·75

The water percentage has risen, and the fat fallen to a very low level.

February (Table X).—Practically all the fish sent during this month were found to be spent, and in even poorer condition than those obtained in January. As they were obtained from the same place as the January fish, the two series are readily comparable.

TABLE X.—FEBRUARY

	Length cm.	Girth cm.	Weight gm.
	26	11	115
	28	12	143
	28	12	155
	29	13	156
	29	12	150
	28	12	136
Average	28 cm.	12 cm.	142·5 gm.

I.—*Muscles.*

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
78·97	18·05	0·68	0·73

(*b*) Per average fish.

Protein	Fat	P_2O_5
16·60	0·62	0·66

II.—*Ovaries* (spent). The average weight of the ovaries per fish was 1 gm.

(*a*) In percentages.

Water	Protein	Fat	P_2O_5
82·07	3·78	11·83	0·91

(*b*) Per average fish.

Protein	Fat	P_2O_5
0·29	0·09	0·016

From these analyses it is seen that the fish had used up practically all their store of body fat. The muscles contained a very high percentage of water, and were in the poorest condition.

The spent ovaries showed the characteristic high percentage of fat seen in that condition, in this case an extremely large amount being present.

It was impossible to obtain West Coast herrings in March, but I examined fish caught in April, and they were practically in the same condition as those taken in May, and of which analyses have been given.

Before studying the changes in the chemical composition of the muscles and ovaries, as shown in the preceding tables of analyses, it is important to note certain points of general interest with regard to the growth of the ovaries.

If one arranges the weights of the fish and ovaries in such a table as the following, one gets a clearer idea of the various periods in the life history of the herring. (Table XI).

TABLE XI.—RATIOS OF WEIGHT OF FISH TO WEIGHT OF OVARIES AT DIFFERENT SEASONS

Period		Weight of fish		Weight of ovaries	Condition
May	...	434·6	:	1	Immature
May	...	301·6	:	1	"
July	...	224·2	:	1	"
September	...	84·2	:	1	" (Ova 0·15 to 0·45 mm.)
October A	...	60·2	:	1	" (Ova 0·28 mm.)
October B	...	20·8	:	1	" (Ova 0·59 mm.)
November	...	8·9	:	1	Almost mature (ova 0·8 to 1 mm.)
December	...	6·3	:	1	Mature (ova 0·9 to 1·2 mm.)
December	...	6·3	:	1	Spawning " "
January	...	8·5	:	1	" " "
January	...	—		—	Spent
February	...	—		—	"

It is interesting to note that the main growth of the ovaries takes place after the most active feeding period is over.

These fish, which spawn in January and February, have their principal feeding time between April and September.

From April to June they feed mainly on copepods, from June to September on schizopods.[1] Food is, however, taken also during October, November, and December, but in smaller amount.

The spawning, according to Brook and Calderwood, takes place six to eight months after the period of richest feeding.

There is no doubt that with the increase in the development of the genitals the desire for food diminishes until spawning time arrives, when no food is taken.

The most interesting period to study carefully is that included under the October, November, and December series.

If one select from each of these tables the fish that are evidently comparable as regards length and girth, namely, in the October Series B the two fish 29·5 cm. in length, in the November series the three fish 30 cm. in length and 16 to 16·5 cm. in girth, and the three 30·5 cm. fish in the December series, one notices that there is certainly no loss in the weight of the fish, but rather a gain as the season advances. That is to say, during the period when the greatest increase in the development of the ovaries takes place, there is no evidence of this growth of the reproductive organs occurring entirely

1. Brook and Calderwood, 'Report on the Food of the Herring,' *Fourth Annual Report, Scottish Fishery Board*, 1886, *Appendix F*, No. VI. pp. 102-128.

at the expense of the other tissues, seeing that there must be in addition some combustion of food material stored in the tissues to cover the energy requirements. There can be no doubt that during this period the fish is beginning to use up its store of fat instead of increasing it as was the case only a short time before.

That there is a distinct loss in the total fat of the muscles of the December fish compared with the October ones is shown by again comparing the amounts of this constituent present in fish of similar size caught during these months. Taking again the same fish from the October, November, and December series as was done for the comparison of weights, one finds that the total fat content of the muscles in the October fish is 20·56 grammes, in similar November fish 19·57 grammes, and in December fish 14·99 grammes. There can be no doubt that this loss of fat cannot be accounted for by a transference to the growing genitalia. The probability is that it is being used to furnish the necessary energy required for the work which the fish is performing.

The Nature of the Chemical Changes in the Muscles of the Herring

The Water Content of the Muscles.—The percentage of water in the muscles rises when spawning commences, and reaches its maximum in the spent fish.

There are, however, exceptions to this rule, as, for example, in the case of the late December fish. The lowest water percentage is, as a rule, to be found when rapid growth of the ovaries is taking place.

Protein.—The changes in the amount of protein are difficult to follow. This may be due in part to the fact that it really was the total nitrogen which was estimated in the case of the analyses given in the preceding tables, or it may be due to the variations in the water content. In the first place, the lowest protein percentage is found at the time when the fat is at its highest level.

There seems to be a gradual transformation of protein, or possibly glycogen, into fat when the stage of active feeding passes into that

of reproductive activity. The average protein percentage between May and September is approximately nineteen, while in October it is fourteen. During the period of rapid growth of the ovaries, and before full maturity, the protein percentage again rises, being due probably to the loss of water from the muscles during this time.

Fat.—Reference has already been made to the variations in the amount of this constituent, but it is necessary to refer to them in more detail.

It is the one constituent which shows regular alterations in its amount during the various stages of reproductive activity, although it is quite possible that glycogen may show similar changes.

It has, however, not been possible up to the present to carry out a satisfactory series of glycogen determinations at the various periods of reproductive activity. This may be said, however, that the amount of glycogen in the spent fish was extremely small in the analyses which I have made.

During the months of May, June, July, August, September, and the early part of October there is a gradual increase in the percentage amount of fat in the muscles.

This increase is most marked from August up to the beginning of October.

It is most likely that during the summer months, when the young ova are just beginning to appear, the food material is mainly stored in protein form in the muscles, while in late autumn the main storage form is fat. In November and early December, when the ovaries are increasing most rapidly in size, the fish falls back upon its store of muscle fat to supply the necessary amount of energising material. By the time that active spawning has commenced the fat shows a great decrease, and in the spent fish of February it reaches its minimum.

The reason why there is not a more rapid fall in fat during October and November is that the fish during this time is still feeding, although, probably, not to the same extent as at the earlier periods. During the process of spawning the feeding ceases altogether.

There can be no doubt that the fish feeds voraciously during

the summer months, hence the frequent occurrence of distended stomach and intestines in fish with immature ovaries. This may lead to the abnormal condition called by fishermen on the West Coast of Scotland 'gut-poke.' Such fish rapidly undergo decomposition owing to the deficient absorption of the intestinal contents. This condition is never found in fish with mature ovaries.

It is interesting to notice the ratios of protein to fat in the muscles at the various seasons. The following table gives these ratios. (Table XII.)

TABLE XII.—PROTEIN : FAT IN THE MUSCLES

Period		Protein		Fat	Condition	
May	...	2·61	:	1	Immature	
May	...	3·00	:	1	"	
July	...	2·48	:	1	"	
September	...	1·63	:	1	"	(Ova 0·15 to 0·45 mm.)
October A	...	0·89	:	1	"	(Ova 0·28 mm.)
October B	...	1·16	:	1	"	(Ova 0·59 mm.)
November	...	1·83	:	1	Almost mature (ova 0·8 to 1 mm.)	
December	...	2·51	:	1	Mature (ova 0·9 to 1 mm.)	
December	...	8·36	:	1	Spawning	
January	...	9·36	:	1	"	
January	...	12·70	:	1	"	
February	...	26·54	:	1	Spent	

Phosphorus.—The variations in the amount of phosphorus are somewhat irregular and difficult to account for.

In October and November fish with rapidly increasing ovaries the average percentage is approximately 0·56 P_2O_5, while during the preceding months from May to September it may be taken as 0·68.

The peculiarly low percentage present in the July fish is difficult to account for. When the ovaries have reached maturity, and also after spawning, the P_2O_5 percentage in the muscles again rises.

CHEMICAL CHANGES IN THE OVARIES

The ovaries of the fish examined showed an increase in size from July to December, but as there were great variations in the size of the fish caught at the various seasons, it is more important to study the amount of the various ovarian constituents per 100 grammes of muscle rather than per average fish.

The following table gives this information :—

Table XIII.—Ovarian Constituents per 100 Grammes Muscle

Period		Protein	Fat	P_2O_5
July	...	0·074	0·044	0·0044
September	...	0·318	0·131	0·0206
October A	...	0·425	0·342	0·0103
October B	...	1·625	0·343	0·0625
November	...	4·193	0·477	0·1477
December	...	6·040	0·675	0·2400
December	...	6·523	0·799	0·2621
January	...	4·286	0·638	0·1621

There is thus a gradual increase of these constituents until spawning occurs. When we compare these figures with the percentage composition of the muscles at the same seasons, one notices that during the time when the protein of the ovaries is showing a distinct increase—for example, from November to December—there is no corresponding decrease in the muscle protein. It is true that between September and October there is a fall in the muscle protein percentage, but it is more readily accounted for by the local increase in fat than by a withdrawal of protein to the genitals.

The herring is thus in a different position from the salmon, at least if one compares the salmon caught in the estuary of a river with those caught in the upper reaches.

The salmon during its sojourn in the river abstains entirely from food. If one, however, studies the composition of the early and late estuary salmon examined by Nöel Paton, one notices that there are but slight variations in the protein percentage and in the total amount per standard fish, whether the ovaries be immature or mature.

The herring undoubtedly does feed practically until spawning occurs, although probably much less food is taken in the later months.[1]

1. Scott, 'The Food of the Herring,' *Scottish Fishery Board Reports*, Part III, p. 260, 1907.

There can, therefore, be no doubt that the herring is not entirely dependent upon its muscle proteins for the growth of the ovaries.

The appearance of fat in large amount in the ovaries of spent fish is similar to that noted by Miescher in spent salmon. This formation of fat was regarded by Miescher as of great nutritive value to these fish.

Male Herrings.—Somewhat similar changes take place in the muscles of the male fish, the highest fat percentage being found just before rapid growth of the testes takes place.

During the later period the fat percentage falls just as in the female.

Composite tables are subjoined, which enable readily a comparison being made between fish caught at various seasons.

Before giving these tables one may shortly summarise the results of the investigation into the life-history of the herring by dividing up the year into the following three periods :—

1. *The Restitution or the Feeding Period.*—This continues for three to four months after spawning, and constitutes the principal feeding time. The spent, thin fish recovers, and accumulates during this time a large store of fat.

2. *The Ripening or Maturation Period.*—This continues for six to seven months. The herring still takes food, but gradually with less desire, and the sexual organs increase as the store of muscle fat diminishes.

3. *The Spawning Period.*—This continues probably for about two months, and during this time feeding stops. Hence there results a very great fall in the fat, accompanied by an increase in the water content of the muscles.

Heincke (*Naturgeschichte des Herings*, p. 48) has shown that the herring after spawning seeks a place where it can get ample food to recuperate. For example, the herrings of Schley, after leaving the spawning beds in June, take three to four months to feed up in Kiel Bay. In September and October they are fattest, and then begins

anew the development of the reproductive organs, which up to this time was checked. This takes up the whole autumn and winter, the fish still taking food, but not using it for the building up of fat, but for the development of the genitalia. With the increase in the development of the genitals the desire for food diminishes until spawning time arrives, when no food is taken.

Hence Heincke, although basing his statements on the life-history of the herring simply on general observations, comes to conclusions which are practically identical with those arrived at by me from a study of the chemical composition of the herring at various seasons.

COMPOSITION OF MUSCLES AND OVARIES IN HERRINGS (Average Fish of Each Series)

Date	Per Fish					Muscle. Per Cent. in Fresh Muscle				Muscle. Per Average Fish			Ovaries. Percentages				Ovaries. Per Fish		
	Lgth.	Girth	Wght.	Wt. of Ovars.	Size of Ovs.	Water	Protein	Fat	P_2O_5	Protein	Fat	P_2O_5	Water	Protein	Fat	P_2O_5	Protein	Fat	P_2O_5
	cm.	cm.	gm.	gm.	mm.														
May	22·9	10·7	90·4	0·208	v. immat.	72·69	18·98	7·25	0·68	11·38	4·35	0·40	—	—	—	—	—	—	—
May	21·3	9·4	72·4	0·24	,,	73·01	17·55	5·85	0·82	8·47	2·82	0·39	—	—	—	—	—	—	—
July	22·9	11·9	100·9	0·45	,,	71·60	18·18	7·32	0·45	12·18	4·90	0·30	72·5	13·52	8·92	0·77	0·05	0·03	0·003
September	20	—	80	0·95	0·15-0·45	63·68	19·28	11·81	0·64	10·41	6·37	0·34	66·02	18·91	7·34	1·23	0·17	0·07	0·011
October, Series A ...	25	13	144·5	2·4	0·2 -0·3	69·97	12·78	14·25	0·53	12·39	13·82	0·51	78·21	17·26	2·53	0·71	0·41	0·33	0·01
,, ,, B ...	27	14	192	9·2	0·4 -0·5	70·46	14·84	12·70	0·57	18·99	16·25	0·73	68·02	22 45	4·80	0·93	2·08	0·44	0·05
November	30·8	16	264	29·5	0·8 -1	66·34	19·87	10·85	0·59	34·97	19·09	1·03	68·04	25·04	2·85	0·91	7·38	0·84	0·26
December, Series A	30·7	16·5	299·8	47	0·9 -1·2	67·36	20·56	8·18	0·68	41·12	16·36	1·37	67·33	25·72	2·89	1·03	12·08	1·35	0·48
,, ,, B	29	14·5	194·5	30·5	0·8 -1·2	72·5	23·01	2·75	0·77	28·76	3·43	0·96	65·73	27·76	3·34	1·19	8·46	1·02	0·34
January, Series A ...	26	13	148	17·4	0·8 -1·4	74·12	18·91	2·02	0·66	18·15	1·93	0·61	70·00	24·75	3·72	0·93	4·23	0·63	0·16
,, ,, B ...	25·5	12	146·5	—	Spent	75·30	19·69	1·55	0·77	18·53	1·51	0·75	—	—	—	—	—	—	—
February	28	12	142·5	1·26	Spent	78·97	18·05	0·68	0·73	16·60	0·62	0·66	82·07	3·78	11·85	0·91	0·29	0·09	0·016

MALES—Average Composition of Muscles and Testes

Date	Lgth.	Girth	Wght.	Weight of testes	Muscle. Per Cent. in Fresh Muscle: Water	Pro-tein	Fat	P_2O_5	Muscle. In Total Muscles of Average Fish: Pro-tein	Fat	P_2O_5	Testes. In Percentages: Water	Pro-tein	Fat	P_2O_5	Testes. Total in Testes per Average Fish: Pro-tein	Fat	P_2O_5
	cm.	cm.	gm.	gm.														
September	20	—	75	0·97	61·68	18·65	14·25	0·52	9·32	7·12	0·25	72·00	22·18	2·84	—	0·21	0·027	—
October	26·7	13·6	166	24	68·91	16·18	12·63	0·50	17·91	13·98	0·55	72·13	22·62	2·25	0·86	5·42	0·54	0·20
November	30·7	16	265·5	38·9	68·22	17·94	10·84	0·46	31·77	19·18	0·81	—	—	—	—	—	—	—
December	31	17	309	58	68·31	21·45	9·24	0·73	44·18	19·03	1·51	72·10	22·05	3·73	2·10	12·78	2·16	1·21

THE MICROCHEMICAL CHANGES OCCURRING IN APPENDICITIS, WITH A NOTE ON THE INCIDENCE OF THE DISEASE IN VARIOUS COUNTRIES

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In observations previously recorded[1] I have shown that intestinal sand and appendix concretions are allied in chemical constitution, and also that abnormal bodies sometimes found in the intestine and producing symptoms of disease are formed of compounds of saturated fatty acids.

These observations led to the conclusions that there are conditions in the intestines in which certain soaps or other insoluble compounds of fatty acids are not absorbed by the intestinal mucous membrane, and that with the presence of these conditions certain diseases of the alimentary tract are related. The fats and soaps found in these conditions were compounds of saturated fatty acids. The richness of these bodies in calcium and fatty acids which are constituents of the excretion of intestinal mucous membrane point to their originating in the intestine as a result of some excess in excretion or defect in absorption.

Recently I have had opportunities of observing further cases in which appendicitis has been associated with mucous colitis or with intestinal lithiasis. In one case of appendicitis with abscess, both before and after operation intestinal sand was found in the faeces in large quantity. In another case, a female who had been operated upon for appendicitis, there was present marked mucous colitis, and intestinal sand was found in fair quantity.

This contribution records further observations on the relation of abnormal fat processes to the aetiology of appendicitis, and shows that similar changes occur in the wall of the appendix itself.

1. 'Abnormal Fat Assimilation,' etc., *Bio-Chemical Journal*, Vol. II, p. 395, 1907.

As far back as 1813 Weigeler[1] examined several stones removed from the appendix of a boy eighteen, who died with all the symptoms of appendicitis. He regarded the stones as biliary calculi which had undergone change from the action of intestinal secretions, and he subjected them to chemical analysis, the results of which were as follows :—

Lapidis granum 1 constabat e.	
Materia pingua adiposa	0·60 gramme
Phosphat calcis	0·30 ,,
Materia animali...	0·08 ,,
Ponderis diminutio erat	0·02 ,,
	1·00 gramme

Rokitansky in 1855 considered catarrhal inflammation of the appendix to be due to faecal concretions, and he believed that this inflammation might persist as a chronic morbid condition. Since then the fleeting references to these concretions are unfortunately the result of only cursory examination, as is shown by their being called faecal concretions, or even the stones of various fruits.

I have had the opportunity of examining a large number of concretions, and not one have I had the slightest difficulty in cutting and of seeing that they are all concentrically laminated, and, therefore, formed in the appendix itself.

Vallee and Bryant[2] state that they have found faeces in 70 per cent. of appendices examined.

I have examined the appendices of 100 consecutive post-mortems in adults, and in only three was true faecal material present ; in the remainder there was a dark slimy mucoid material, in some cases not unlike faeces in consistence, but not showing the same pigmentation as true faeces.

Expressing this material into alcohol I have been able to isolate a small amount of fat from it, and this gave a low iodine value (the quantity examined was too small to give an accurate figure). The nature of the fat thus resembles the fat isolated from the loop of intestine in the case quoted in the previous paper.

1. Quoted from Kelly's *Vermiform Appendix and its Diseases.*
2. *Ibid.*, p. 148.

I was fortunate enough in one case to find in the appendix small granules looking exactly like intestinal sand, showing the likeness not only in nature but also in the formation of this substance and the appendix concretion.

The frequency of these concretions is undoubted. Deaver[1] gives the following figures :—

Variety	Percentage of class of case	Percentage of presence of calculus
Acute Catarrhal	3·7	None
Acute Interstitial	15·9	6·2
Acute Ulceration	59·4	22·3
Acute Gangrenous	20·9	10·2
Chronic Catarrhal	1·5	Rare
Chronic Obliteration	1·5	None
Chronic Interstitial	0·97	1·55

The great frequency of occurrence points to some close relationship to the disease, and as previous writers have considered them from the standpoint of their being inspissated faeces, it will be of advantage to consider their formation in the light of their constitution as revealed by the following observations.

I have shown[2] that they are undoubtedly formed in the appendix, and Kelly states :[3] 'The histological examination shows that the most important factor in the formation of the calculus is the mucus secreted by the glands of the mucous membrane. The mucus, which is deposited in layers round the central nucleus, becomes desiccated, and the lime salts are deposited secondarily. Frequently fragments of epithelium, a few leucocytes, and altered blood are found in the different layers. Ribbert noted that in favourable specimens stained with Weigert's fibrin stain, the outer layers of mucus may be seen to be directly continuous with the glands of Lieberkühn. In one case, besides the two large enteroliths in the canal of the appendix, *I found three smaller bodies embedded in the mucous membrane, which had evidently developed within gland lumina.*'

This latter statement is full of meaning in view of my findings, to be described later.

1. Battle and Corner, '*The Surgery of Diseases of the Appendix.*'
2. 'Abnormal Fat Assimilation,' *Brit. Med. Journ.*, July 27, 1907.
3. *Loc. cit.*, p. 300.

Kelly gives further evidence of this mode of formation of the calculus.[1]

Concretions do not occur in all cases of appendicitis, but the following investigations into the nature of the changes taking place in the appendix show that the process by which they are formed may go on in the wall of the appendix and give rise to morbid conditions which allow of easy invasion by micro-organisms, and thus to the acute and chronic forms of appendicitis. These changes are the formation of calcium soaps in the submucosa and mucosa of the appendix. These soaps, normally expelled into the lumen of the appendix and then into the caecum, in cases of appendicitis block up the lumen of the glands or (as in many of the specimens obtained) form a ring in the submucosa.

The micro-chemical tests which I have employed are the same as those used by Klotz[2] and Lorrain Smith.[3]

These methods depend upon the following chemical facts :—

Osmic acid stains neutral fats black; it does not blacken palmitic and stearic acids.

Sudan III, scharlach R, and other similar bodies stain neutral fats a bright pink, whereas they stain soaps a pinkish yellow. The difference in these two colours is easily seen in the specimens obtained.

Nile-blue sulphate stains neutral fats pink, and stains fatty acids violet.

Silver nitrate forms an impregnation of silver oxide on calcium compounds.

Petroleum ether dissolves neutral fats from a specimen, but not soaps. This can be used as a further test for differentiating these two compounds.

1. *Loc. cit.*, p. 301.
2. *Journal of Experimental Medicine*, Vol. II, No. 6, Nov. 25, 1905.
3. *Journal of Pathology*, p. 283, 1907.

THE MICRO-CHEMICAL CHANGES IN THE WALL OF THE APPENDIX

Sections of appendices stained by the above methods show the colour reactions from which the nature of the chemical changes in the wall have been deduced. The specimens were obtained from appendices removed by operation and placed immediately in formol.

Post-mortem specimens have been examined, but as the primary effect of autolysis is fatty degeneration, the true condition of the cells in life is not to be obtained from such specimens.

Zuckerkandl[1] has shown that, as the result of age, the mucous membrane undergoes atrophy, and the glandular structures desquamate. At the same time, or occasionally before this time, the submucous coat undergoes thickening, and *fat* accumulates at this part of the wall.

A normal appendix from a child, obtained at operation, is required as a standard. This I have not been able to obtain, and failing this, I have compared my sections with those of an appendix (apparently healthy) of an adult, removed from a hernial sac. This abnormal position would possibly interfere with its function to some extent, but nevertheless the specimen shows little fatty degeneration of the mucous membrane, and only traces of soaps in the submucosa. These slight changes may be considered to be due to either normal involution changes or to the abnormal position of the appendix.

Stained to show the calcium present, there is some to be seen in the mucous membrane at certain points. Sections from diseased appendices treated by the same methods and under the same conditions show most striking differences: differences which are so great as to be immediately obvious in sections stained in bulk and observed macroscopically. The changes cannot be due to involution changes, as they are seen in specimens obtained from cases occurring in children.

These sections show a *large increase in the production of calcium soaps,* both in the mucous membrane and in the submucosa. In some of the specimens almost a complete ring of soaps is to be found in the submucosa.

1. Nothnagel, *Diseases of the Intestines and Peritoneum*, 1905, p. 382.

A change in the submucosa has been frequently recorded as a marked thickening. This thickening is in the above specimens seen to be due to the laying down of these soaps. Small quantities of fatty acids are also found in the submucosa by the Nile-blue sulphate method.

Staining by silver nitrate shows the presence of calcium in definite quantity in the submucosa of the diseased appendices.

These observations, therefore, show that in appendicitis there is a marked change in the wall of the tube—a change characterised by the increased production of calcium soaps in the mucous membrane, and more particularly their formation in the submucosa.

A few of the specimens show the secretion or rather excretion of these soaps into the lumen, and thus confirm Ribbert's observation that it is the mucus, as he called it, which is poured forth which forms the concretion. This is further proved by the fact that the chemical constitution of the excretion and concretion is the same.

That this process can, in rare instanccs, go still further is shown in a case recently published.[1] A man aged forty-one had his appendix removed for an acute attack of appendicitis. The appendix was unusual in shape, and opened into the caecum by a very wide orifice. Distal to this orifice was a marked hypertrophy of the muscular layers. This extended for about half the length of the tube. Beyond was a dilated portion full of white pultaceous material. Some of the material was examined qualitatively and shown to contain calcium soaps and calcium carbonate, the latter apparently in fair quantity. The calcium carbonate was formed as the result of further degeneration of the calcium soaps.

The orifice of this appendix was so abnormally wide that it is inconceivable that the material could arise from the intestine, as there was such free drainage, and the offending material had accumulated behind the hypertrophied muscle.

These observations show that in appendicitis there is a change in the nature of the fatty compounds far in excess of any I have so far been able to observe as the result of involution changes.

1. *Brit. Med. Journ.*

The important question consequently arises whether this change is the cause of the disease, or whether there is some underlying cause which produces these changes and the disease. From what we know of the formation of these calcium soaps, it is a very slow process, and, therefore, must have commenced long before the attack of acute disease. It may, therefore, be presumed that these fat changes go on, produce the altered condition of the mucous membrane, or in some instances form an impermeable barrier in the submucosa, cutting off the nutritive supply to the already altered mucous membrane. The invasion by organisms is then rendered much easier.

The nature of the fats in the food determines to some extent the nature of fats in the tissues. It would, therefore, seem that as these abnormal processes are set up by saturated fatty compounds (palmitic and stearic) and not by unsaturated fatty compounds (oleic), the nature of the fat in the diet might have some influence in their production. This is made still more likely when we recognise that the absorbability of these two forms of fat is so different.[1]

Incidence of Appendicitis

I have, therefore, endeavoured to find if the nature of the food fats has any relation to these pathological processes—whether an excess of saturated fat (as in beef and mutton) in the diet is more liable to produce the disease than unsaturated fat (as in olive oil, etc.). I wrote to His Majesty's representative in all countries having a population of over two millions, and from a large mass of correspondence I quote the following which have relation to the subject.

For comparison it may be stated that the death-rate in England from appendicitis in 1902 was 90 per million persons living.

Spain.—Dr. Glendinning writes : 'The facts are, firstly, that it is comparatively rare. In my occasional visits to various hospitals during the year I have not seen a single case of appendicitis. Secondly, it is slightly more common among the aristocratic classes. Dr. Guttieriez, who has the largest consulting practice in Madrid, informs

1. *Brit. Med. Journ.*, July 27, 1907.

me that he has personally seen four cases in the last year, and has heard of another three as being seen by colleagues.'

Examining the statistics, I find that the death-rate from 'appendicitis and inflammations of the iliac cavity' is twenty per million.

Dr. Glendinning states that there is not as much oil in the diet of the Spanish people as is commonly supposed.

Italy.—An Italian doctor visiting the Royal Infirmary, Liverpool, stated that the disease was comparatively rare in Italy, but Professor Bastianelli writes that the disease is common in Rome and that part of the country. The statistics of operations which Professor Bastianelli sends me show that an average of 156 cases are operated on in Rome annually from a population of 500,000.

This would give the incidence as far less than that of Liverpool.

Sweden.—The death-rate is about 16 per million. The consumption of meat is about 59 lbs. per head per annum, or about 2½ oz. per diem, which is less considerably than in the English diet.

Denmark.—The death-rate is about 12 per million. 'It may be said, however, that the staple diet will be about the same as that of Great Britain, only that less meat and vegetables are eaten, whereas more potatoes, rice, and other starch-containing articles are consumed.'

Cairo.—Out of 13,671 cases admitted to the Kaisr-el-Ainy Hospital there were *only fourteen* cases of appendicitis. This is an extremely low percentage compared with English hospitals.

The registrar states that beans cooked in oil is a very common dish among the natives.

Iceland.—The chief physician writes: 'The disease is in my own and other medical officers' opinion of the same comparative occurrence here as in other countries. Fats are consumed in a higher degree than among common people in most other countries.'

Unfortunately the nature of the fats is not stated, but it is safe to assume that meat fats are meant.

Belgium.—Fat is taken in large quantity, particularly in the form of butter (which contains a good deal of *unsaturated* fat—of *oleic* acid), lard, and margarine.

Dr. Nicoleb writes ‘that it is his own impression that appendicitis is not a frequent disease.’

Greece.—Out of 28,067 cases admitted to hospitals, there were only 393 cases of appendicitis (a figure far less than that given for the Liverpool hospitals).

‘A good deal of the cooking is done with olive oil and butter.’

Norway.—‘The disease is very frequent in this country. It seems to be more so in the greater towns than in the rural districts.’

‘With more prosperous circumstances and a more liberal diet the frequency seems to increase.’

‘Safe statistical statements as to the diet do not exist.’

China.—‘Appendicitis is an extremely rare disease in China.’

‘Some time ago inquiries made through the *China Medical Journal* among all the medical practitioners show that forty-four had rarely seen the disease, sixty-nine had seen a few cases but thought the disease uncommon, forty-nine had not seen any cases at all.’

‘In the Public Mortuary, out of 2,140 post-mortems, appendicitis is not once mentioned, nor does it occur in the list of 215 surgical operations performed in the Government Civil Hospital.’

‘Foreigners resident in China have no immunity from the disease.’

Diseases of the alimentary system are *more common* than those of all the other systems.

‘The system of diet, bolting of food, etc., are the main causes of the frequency of digestive troubles.’

‘It will thus be seen that there are two outstanding points of difference between the Chinese and European dietaries, viz., *the lessened quantities of sugar and meat in the former scale.*’

‘Meat is eaten fresh, and *there is an entire absence* of tinned and frozen meats, which by their cheapness and adequate supply have increased the consumption of meat per head during the past decade or two in Europe—especially in England.’

'No one here has been able to offer, as yet, any feasible explanation of the freedom from appendicitis, though most doctors think *there must be a predisposing cause in the European diet.*'

India.—I have no statistics personally, but quote from the *Lancet*, April 18, 1908, p. 1,162: 'It seems to be clear that appendicitis is *rare* in India.'

Mr. James Harris, L. M. and S., the writer ot the article in Madras, suggests the simple diet of the people as the cause of their comparative immunity. He further suggests the frequent habit of purgation and the mode of defaecation as factors.

Lewkowitsch[1] states that linseed and rape oils (markedly unsaturated) are largely taken in India.

Abyssinia.—The Medical Officer of the British Legation states: 'During a residence of eight years or so in this country, and a practice among all classes of the population, I have never come across a single case of appendicitis, nor have my colleagues ever met with what could be called an authentic case of the above malady. . . . . A considerable amount of melted butter is the principal fatty constituent of their diet.'

Holland.—Statistics obtained from Dr. C. Eykman show that the incidence in this country is about the same as in England—from 88 to 113 per million. It is more frequent among males. He states that 'as everywhere else the diet of the well-to-do classes differs from the poorer classes in that the former consume a greater quantity of meat, and in that connection of albumen and fat.'

England and America.—There seem to be two definite facts about the disease in these countries: first, that it is more frequently a disease of the rich than of the poor; and second, that it is on the increase.

There is no doubt that in these countries meat is eaten in large quantity, and that the facilities for obtaining meat are much better now than a few decades ago, when the disease was much less common.

The above statistics would tend to show that the incidence of

1. *Chemical Technology of Oils and Fats*, Vol. II, 1904.

the disease is greater in those countries where much saturated fat is consumed.

As the statistics of this disease are as yet in all countries incomplete, it is difficult to draw deductions from them, but they are worthy of consideration.

Conclusions

From clinical evidence and pathological and chemical observations it would appear that there is produced in the course of the changes in the intestinal wall an abnormal condition associated with formation of calcium soaps. These calcium soaps when formed in the intestine in excess produce intestinal sand, and associated with this there are symptoms of colic with, under some circumstances, an associated form of mucous colitis, or, as the French authors call it, muco-membranous enterocolitis.

This change taking place under similar conditions and probably from the same causes in the appendix, where there are poorer opportunities for the discharge of the deleterious bodies, is probably an important factor in the aetiology of appendicitis.

The cause of these pathological changes is possibly an excess of saturated fats in the food-stuffs, giving rise to the formation of calcium soaps of more saturated fatty acid in the mucosa and submucosa than is normally the case. These soaps are not so easily absorbed as the soaps of the unsaturated fatty acids, and, therefore, act as foreign bodies in the wall, and at times in the lumen as a concretion.

My thanks are due to Sir James Barr for valuable aid in the collection of statistics, the physicians and surgeons of the Royal and Children's Infirmaries for many specimens, and to Professor Benjamin Moore for his constant advice during the conduction of these researches.

THE ACTION OF PILOCARPINE ON THE HEART

(Preliminary Communication)

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During certain months of the year the vagus nerve has little or no inhibitory power over the amphibian heart. This important functional variation has been noted by several workers, and has been employed as a means of deciding the vexed question of the action of pilocarpine and muscarine on the heart.

If pilocarpine or muscarine has no action on a heart whose vagus is without inhibitory power, and can slow or diminish the force of or stop a heart only when the vagus is active, one would naturally conclude that pilocarpine or muscarine acts on the vagus nerve endings (the cardiac ganglia being for the present left out of account). G. N. Stewart, in his *Manual of Physiology* (Edition 1895, and subsequent editions up to 1906), emphasises this point. 'It is quite in accordance with this' (*i.e.*, the view that muscarine acts on the vagus fibres between the nerve cells and the muscle or the actual nerve endings) 'that muscarine has no effect on a heart whose vagus nerves, as occasionally happens, have no inhibitory power. Pilocarpine has very much the same action as muscarine.'

More recently, this field has been thoroughly developed by MacLean (*Bio-Chemical Journal*, Vol. III, Nos. 1 and 2). He concludes that 'the effect produced on the heart by small doses of muscarine and pilocarpine is in direct proportion to the influence exercised on the heart by the vagus nerve.'

Marshall further pointed out that an immunity to pilocarpine could be induced by repeated dosage (*Journal of Physiol.*, Vol. XXXI, p. 120, 1904). This was true in mammals (Marshall) and in the frog (MacLean).

The writer, working on the amphibian heart (frog, newt and salamander), finds :—

I. In the newt and salamander, the heart may be primarily immune to pilocarpine (*i.e.*, an immunity not due to dosage). Yet the heart can be inhibited on faradisation of the sinus with certain strengths of current.

II. In the frog and salamander, during the immunity to pilocarpine induced by administration of that drug, similar faradisation of the sinus can inhibit the heart.

III. In the frog and salamander, when a heart slowed by early doses of pilocarpine is quickened or enforced by a renewed application of the drug, the revival of cardiac activity does not mean that the heart can no longer be inhibited by faradisation of the sinus.

IV. In the frog, when a heart brought to a standstill by the application of pilocarpine revives naturally or is made to beat again by experimental manipulation, in both instances faradisation of the sinus can lead to a greater or a less degree of inhibition of the now immunised heart.

V. In the salamander, the heart found primarily immune to certain strengths of pilocarpine may be rendered by experimental manipulation susceptible to a dose formerly unable to influence it. Then, later, this dose loses its power over the heart. Throughout, and at the close of the experiment, faradisation of the sinus inhibits the heart.

This cardiac inhibition following on faradisation of the sinus may be due to—

A.—Stimulation of the inhibitory nervous mechanism of the heart. Such an explanation is, no doubt, most in line with current belief. Assuming that it is correct, then the theory that pilocarpine acts on the nerve endings of the vagus must be revised.

One is led in this case to consider the possibility of pilocarpine acting on the heart by virtue of a combination with some substance in the cardiac tissue. A natural immunity to pilocarpine might then be due to the failure of this combination to take place or to the absence of this substance. Immunity produced by repeated doses

of pilocarpine might be explained by supposing that the combination between pilocarpine and this specific substance has been rendered inert.

But inhibition following on faradisation of the sinus may be due to—

B.—The direct action of the induced current on the cardiac tissues independently of inhibitory nerves. If such should prove to be the correct interpretation, then present views as to the reaction of the cardiac tissues to the faradic current require revision.

The writer reserves meanwhile the evidence at his disposal bearing on either view (A or B). It is sufficient to indicate at this stage certain important questions raised by this inquiry.

THE ACTION OF DRUGS ON MAMMALIAN UTERUS

By HAROLD J. FARDON, M.A., M.D.

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(*Received August 4th*, 1908)

The Nervous Mechanism of the Uterus

The literature on the nervous mechanism of the uterus is fairly complete. In 1858 Lee demonstrated the ganglia in the peripheral plexus surrounding the cervix uteri; and in 1867 Frankenhaeuser described the anatomy of the hypogastric nerve and its associated plexuses; he also demonstrated microscopically terminal, non-medullated and nucleated nerve fibrils situated between the muscle fibres of the uterus and ending in the nuclei of the muscle cells. The descriptive anatomy was verified in 1895 by Langley and Anderson,[1] who stimulated the lumbar roots which supply the uterus. Working chiefly on cats and rabbits, they showed that the uterus is supplied solely by the true sympathetic system. The sacral roots and pelvic nerve do not enter into the composition of the uterine plexus.

Electrical stimulation of the hypogastric nerve, in the rabbit, always produces well-marked contraction of the uterus associated with vaso constriction.

In some cats such stimulation produces contraction of the uterus, while in others relaxation results.

Dale[2] has shown that the use of ergotoxine adds a further facility to the study of this subject, by paralysing the augmentory fibres while leaving the inhibitory fibres untouched. Stimulation of the hypogastric nerve after the administration of ergotoxine produces inhibition of the uterus in all animals, thus demonstrating the presence of utero-inhibitory fibres in addition to utero-augmentory fibres in this nerve. The relative proportion of these two sets of nerve fibres will be referred to when dealing with the action of adrenalin,

1. *Journ. Physiol.*, Vol. XIX, 1895.
2. Dale, *Journ. Physiol.*, Vol. XXXIV, 1906.

and the presence of peripheral nerve cells will be considered with the action of nicotine.

The present investigations have been made entirely on the isolated uterus; the attention has been confined solely to those drugs which act either on the uterine muscle or on some portion of the peripheral nervous mechanism. Under these conditions no interference is possible from alterations in blood pressure or from central excitation.

Method

An isolated mammalian uterus kept in Ringer's solution at a temperature between 30° C. and 38° C., and supplied with oxygen, will live and continue its rhythmical movements for several hours; if it be hung up and attached to a lever these movements can be recorded.

There are, however, certain difficulties to contend with in all investigations on isolated mammalian organs. The chief of these is the necessity for artificial heating. This is especially important in the case of the uterus, because its movements are extremely sensitive to very slight alterations of temperature. It was found, however, that the simple method of applying a bunsen to the end of a metal bar passed through the water bath surrounding the beaker of Ringer's solution was the most satisfactory. Experience soon showed the amount of gas necessary to keep the bunsen at such a height that the Ringer's solution should record a constant temperature of 37·5° C.

The method adopted for recording the movements was as follows :—The upper ends of both cornua were fixed to a lever by means of a thread tied to the ovaries, while the upper end of the vagina, which was detached with the uterus, was fixed to the bottom of the beaker, in which the viscus was perfused with 300 c.c. of Ringer's solution. Sometimes the cornua were separated, the division being made through the longitudinal axis of the vagina. This did not interfere with the activity of the uterus, while it allowed of control experiments being performed on the same animal. A suitable tonus is imparted to the uterus by a carefully-adjudged weight.

Most of the experiments were performed on the uteri of cats and rabbits. Other animals, the bitch and guinea pig, were occasionally used for control purposes. It may be stated generally, that the effect of drugs was similar in all animals used. The animals were killed usually by pithing.

The drugs were added directly to the Ringer's solution.

The oxygen was supplied at a continuous rate of about seventy small bubbles a minute.

Normal Movements

The normal movements of the uterus show considerable variations in different animals, and the degree of activity in responding to physical and chemical stimuli is equally inconstant. In the pregnant uterus, as a rule, the movements are much more pronounced and vigorous than in the non-pregnant uterus. This is principally due to the large hypertrophy of the organ in this condition. On the other hand, the poorly-developed uterus of young animals never gives good results.

The regularity of the movements is disturbed by alterations in the temperature, and sometimes such small changes as 0·2° C. or 0·3° C. produce a definite alteration in the tone of the muscle.

Alterations in the rate of oxygen supply also has an appreciable effect on the uniformity of the rhythm.

In the absence of oxygen the uterus remains inert, and when the oxygen supply is deficient the spontaneous movements are feeble.

One uterus may respond well to certain drugs, while another equally well developed uterus of a similar animal, and under similar conditions, may respond hardly at all. This has led me to suppose that the period of the menstrual cycle at which the experiment is performed may influence the results.

Pregnancy has a special effect on the action of drugs on the uterus. Adrenalin, for instance, by stimulating the sympathetic, relaxes the non-pregnant uterus of a cat, but contracts a pregnant one. This may be due to a positive increase in the number of augmentory fibres during pregnancy, or to a change in the muscle

cells which renders them more responsive to impulses from the augmentory fibres. The uterus of a cat produces the most uniform movements, and, therefore, gives the most satisfactory results. The guinea pig's uterus, besides being fragile, is very erratic, and is the most sensitive to variations of temperature.

The commonest type in all animals is a continuous see-saw movement without intervals. Sometimes a fuller relaxation or a stronger contraction would alternate at regular intervals with smaller movements, at a mean tonus. Or the smaller movements may be absent, and a stage of quiescence, lasting about two or three minutes, is broken by a strong contraction comprising the whole or greater part of the contractile power of the muscle. In these cases the tonus, of course, cannot be altered except by diminution in the amplitude of the contractions. Another type, seen chiefly in the guinea pig's uterus, is a strong contraction followed by a gradual relaxation broken at intervals by small secondary contractions.

These spontaneous movements sometimes commenced as soon as the uterus was attached to the lever. They were usually visible in the animal before removal. Sometimes, however, the shock of removal caused a cessation of the movements, which lasted for a variable length of time, and a careful adjustment of the tonus by alteration of the weights was necessary to initiate them.

A rise of temperature, or an increase or decrease of the oxygen supply, in some cases was sufficient to start them.

In a few uteri, artificial alterations of tonus, produced by moving the lever up and down with the hand, was necessary to initiate spontaneous movements. When once started they usually continued for many hours under good conditions.

Effects of Acids and Alkalies

Alkalies in small amount are essential for the spontaneous movements of the uterus, and for keeping the organ in the best physiological condition. When the alkali is increased the effect is to augment the tonus, while the spontaneous movements gradually diminish, until death of the muscle ultimately occurs at a high tonus. Diminution

in alkalinity of the perfusion fluid lowers the tone of the uterus. When the acid reaction is obtained, the movements are gradually arrested. If the solution be suddenly rendered strongly acid, then a forcible contraction occurs, and the muscle dies in contraction. If, however, the acidity be at once neutralised, the tone and movements may recover. (Fig. 1.) These results may be compared with the well-known effect of acids and alkalies on striped muscle and on the heart.

Drugs Acting on the Nervous Mechanism

In the first series the following drugs are considered :—Pilocarpine, physostigmine, and colchicine. These drugs are all stated to act on 'nerve endings.' They cause augmentation of peristalsis in the small intestine, having a greater affinity for the vagus than for the splanchnics. They ignore the sympathetic supply to the iris and submaxillary gland, and have only a very slight action on the cardiac sympathetic.

After atropine—which paralyses the vagus, or, at all events, those portions of it which are excited by the drugs under consideration, while leaving the sympathetic nerve endings unaffected or only very slightly affected—these drugs are still unable to produce inhibition of peristalsis in the small intestine, showing, apparently, that they do not act on the splanchnic fibres. These drugs do not apparently all act on the same parts of the nerve endings. Physostigmine is capable of constricting the iris after atropine, but not after nicotine. The converse is the case with pilocarpine.[1]

In considering the action of these drugs on the uterus, it is necessary to remember that both augmentory and inhibitory fibres are supplied by the sympathetic, and that the relative proportion of these two sets of fibres varies in different uteri, the preponderating set being readily ascertained by means of adrenalin, as will be described later. By analogy with the intestine, one would expect that the action of this group of drugs would be slight; it would be exerted on both sets of fibres equally, and the result would depend, therefore, upon the predominating set.

1. Dixon and Malden, *Journ. Physiol.*, Vol. XXXVII, 1908.

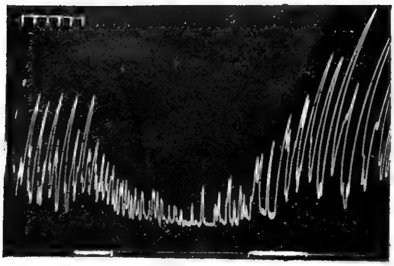

Fig. 1.—Uterus of cat, showing action of acid and alkali. HCl given at the first mark, $NaHCO_3$ given at the second mark. Upstroke = contraction. Time = minutes.

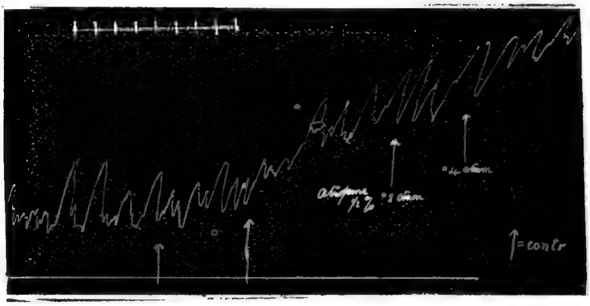

Fig. 3.—Uterus of cat, containing four ova. Colchicine (1·5 mgms.) given at the first arrow, and repeated at the second, raised the tone of the uterus. Atropine (1 mgm. at the third arrow, and 2 mgms. at the fourth) still further increased the tonus. Time = minutes.

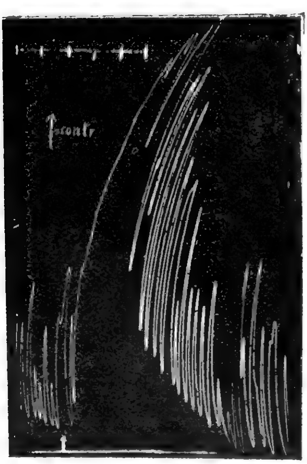

Fig. 2.—Non-pregnant uterus of cat, showing action of pilocarpine (10 mgms.). Upstroke = contraction. Time = minutes.

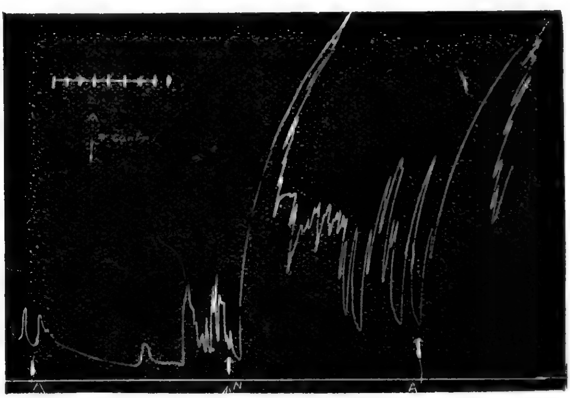

Fig. 4.—Non-pregnant uterus of cat. Adrenalin (1-1000) m i given at the first arrow. The drum was stopped for ten minutes, until the spontaneous movements had recovered, and then nicotine (10 mgms.) was given at the second arrow, and produced relaxation followed by contraction. Adrenalin (1-1000) m i given at the third arrow produced contraction of the uterus. Time = minutes.

Pilocarpine.—To a certain extent this expectation is realised with regard to pilocarpine. The most frequent result with this drug is augmentation of tone with some increase in the amplitude of the spontaneous contractions. (Fig. 2.) This effect is not well maintained.

Relaxation of uterine muscle is very difficult to obtain with pilocarpine. I have, however, observed it after paralysing the augmentory fibres with ergotoxine. In one experiment on a pregnant uterus of a cat, the two cornua were separated, and one was kept in a solution of ergotoxine for half an hour. Pilocarpine produced slight relaxation in the ergotised cornu, and slight augmentation in the other cornu.

The action of this drug on the uterus is completely antagonised by atropine.

Pilocarpine, therefore, has a special affinity for the augmentor fibres of the sympathetic, since in the non-pregnant cat, *i.e.*, when the development of inhibitory fibres is maximum, this drug still augments the movements. The action is clearly on nerve endings, because it is eliminated by atropine, and, moreover, ergotoxine, which paralyses augmentory nerve endings, converts the stimulant action of pilocarpine into a very mild inhibitory one.

Physostigmine.—Physostigmine always augments the movements and the tone of the uterus. Its effect is better maintained than is that produced by pilocarpine, and is produced with smaller doses. This was the case with all animals used, and under all conditions. This augmentation occurred after atropine, and was not apparently influenced by atropine. Physostigmine also produces its effect after nicotine.

This drug is considered to produce a direct stimulant effect on plain muscle in addition to its action on nerve endings (Harnack). It would be expected that this additional property would have greater influence on the uterus than one which depended on sympathetic nerve endings. This would account for its action being better maintained than that of pilocarpine as well as for the fact that its effect is produced after atropine and nicotine.

Colchicine.—Colchicine is purely an augmentor of uterine muscle, and this effect occurs chiefly in rabbits where the augmentory fibres predominate both in pregnancy and non-pregnancy. This augmentation is considerably greater than that produced by pilocarpine. In the cat's uterus the action varies with the condition. In the case of the pregnant uterus in which adrenalin augments the movements, colchicine also raises the tone, whilst in the non-pregnant condition this drug has very little action. An inhibitory effect was never obtained by colchicine even after a previous dose of ergotoxine.

Large doses of colchicine destroy the movements of the muscle. The action of colchicine on the uterus is not antagonised, but rather increased by atropine. (Fig. 3.) This is interesting in view of the fact that atropine antagonises the effect of colchicine on the heart, respiration, and intestines.[1] The action of colchicine on the uterus is, however, prevented by ergotoxine, and, therefore, is produced on the augmentory nerve endings.

Adrenalin.—It has been already abundantly shown that adrenalin excites all sympathetic nerve endings, and that its effect on the uterus in all cases corresponds exactly with that obtained by electrical stimulation of the hypogastric nerve.[2]

It has been further shown that the augmentor fibres of the sympathetic nerve can be paralysed by ergotoxine so that a pregnant uterus, which under ordinary conditions actively contracts with adrenalin, after ergotoxine becomes relaxed.[3]

As I shall show later, my experiments demonstrate conclusively that nicotine also influences the action of this drug so that if adrenalin be allowed to act upon the non-pregnant uterus of a cat, instead of inducing relaxation of muscle as normally, after nicotine it gives rise to marked contraction. The action of adrenalin is of great importance as a means of determining the action of the sympathetic nerves. It has been of much value in ascertaining the relative proportion of the augmentory and inhibitory nerve fibres to the uterus.

1. Dixon and Malden, *Journ. Physiol.*, Vol. XXXVII, 1908.
2. Elliott, *Journ. Physiol.*, Vol. XXXII, 1905.
3. Dale, *Journ. Physiol.*, Vol. XXXIV, 1906.

The augmentor fibres invariably predominate in the rabbit's uterus under all conditions, so that adrenalin produces a rise of tonus. By first paralysing these augmentor fibres with ergotoxine, adrenalin can be used to demonstrate the presence of inhibitory fibres,[1] since under these conditions the uterus relaxes both to electrical stimulation of the sympathetic nerve, and to the administration of adrenalin. In the cat, the results vary with the condition of the uterus. Adrenalin raises the tone of a pregnant uterus, except after ergotoxine. Cushny believes that inhibition by adrenalin occurs in cats only with virgin uteri, but I have obtained it in the uteri of two cats during lactation. Subinvolution of the uterus was, in these cases, complete. In every non-pregnant cat in which adrenalin has been given at the onset of an experiment, I have obtained relaxation of the uterus.

The reverse effect, however, is sometimes obtained on the non-pregnant cat by the use of adrenalin after the administration of other drugs. In three experiments on these animals adrenalin augmented the tonus of the uterus after the administration of nicotine, when the initial effect of adrenalin had been inhibition. In two of these, nicotine was the only drug given previously. In two other experiments, although the initial effect of adrenalin had not been determined, I obtained a rise of tonus in a non-pregnant cat's uterus with this drug after previously administering pilocarpine, atropine, and nicotine. Pilocarpine and atropine alone do not produce this reversion. It appears, therefore, that nicotine has a distinct power to reverse, in some way, this action of adrenalin on the uterus. (Fig. 4.) As adrenalin acts on nerve endings, this power of nicotine must be exerted either on these nerve endings or on the muscle itself. It is not a paralysis of the inhibitory nerve endings, for if ergotoxine be added after the reversion has been effected, the adrenalin action is once more reversed, and produces inhibition.

The relation of nerve endings to muscle is, as yet, insufficiently understood. Nicotine alters this relation so that the muscle is rendered more responsive to impulses derived from the augmentor

1. In one experiment adrenalin produced no result after ergotoxine. This occurred in the pregnant uterus of a rabbit at full term.

nerves, thus turning the balance in their favour. This reversion is not always effected by nicotine; possibly when it does not occur the inhibitory nerves are very greatly predominant.

Stewart,[1] when discussing the effect of stimulation of the hypogastric and pelvic nerves on the bladder, incidentally makes the following statement :—‘ Nicotine seemed to affect the proportion between the rise and fall in the internal pressure of the bladder produced by stimulation of the hypogastric.’

In one experiment adrenalin produced augmentation in the non-pregnant uterus of a cat to which cocaine had been previously administered. The initial effect in this experiment, however, had not been ascertained.

Whether adrenalin produces contraction or relaxation of the uterus, its effect soon passes off. A repetition of the dose then produces the same effect. This is in contrast to the effect produced by nicotine, where the return to the normal tonus is due to depression or paralysis of the nerve following on the stimulation.

Inhibition of the uterine movements by adrenalin usually results in relaxation of the muscle in addition to cessation of movements. In some cases relaxation is absent. I presume that in these cases the spontaneous movements were occurring originally at full relaxation, so that adrenalin was unable to relax the muscle any more. Thus the action of adrenalin is to a certain extent a measure of the tonus at which the spontaneous movements are produced.

Cocaine.—Cocaine augments the tonus of the uterus of both cat and rabbit in all conditions. (Fig. 5.) It probably acts on the sympathetic nerve endings, the effect being analogous to the contraction of the dilator iridis, the inhibition of intestinal peristalsis, and the acceleration of the heart also produced by this drug, evidence as to the seat of action in those cases being fairly complete. Cocaine never relaxes uterine muscle. Ergotoxine does not influence its action, and this might be regarded as evidence that it acts on the muscle, but nerve endings are now believed to be of such a composite nature that it is possible for ergotoxine to antagonise adrenalin while

1. *American Journal of Physiology*, Vol. II, p. 190, 1899.

not interfering with that portion of the nerve ending on which cocaine acts. Cocaine, then, whilst increasing uterine tonus by exciting sympathetic nerve endings, acts differently from adrenalin, because its effect is not removed by ergotoxine. It shows none of the characteristics of a true muscle poison.

Nicotine.—Langley and Anderson,[1] in their experiments on cats and rabbits, showed that some of the sympathetic fibres to the uterus passed by the inferior mesenteric ganglia, and proceeded to more peripheral ganglia on the course of the hypogastric nerve. They also showed that nicotine paralysed these peripheral ganglia as well as the more central ganglia.

Nerve cells are also present in the ganglionated plexus on the walls of the uterus and vagina. They are under the peritoneal coat of the viscus, and were of necessity removed with the uterus in all my experiments.

On an isolated uterus of a cat previously ergotised so that only the inhibitor fibres were responsive, nicotine produced immediate relaxation followed by a gradual recovery of the tonus and of the spontaneous movements. (Fig. 6.) A repetition of the dose produced no further effect, while adrenalin administered after the nicotine still produced its usual action. Nicotine had presumably first stimulated and then paralysed some other more central part of the peripheral inhibitory plexus than that upon which adrenalin acts. A similar result can be obtained with nicotine on an isolated coil of small intestine in which also peripheral nerve cells are present. It seems, therefore, that when nicotine produces a result on isolated viscera that receive their nerve supply from the sympathetic, its action is mainly on peripheral nerve cells. This action is identical with that obtained on more central ganglia by intravenous injection of nicotine in intact animals, viz., stimulation followed by paralysis. This fully explains the result obtained with nicotine on the utero-inhibitory nerves as demonstrated on the ergotised uterus. There is no means of paralysing the inhibitors while leaving the augmentors untouched in order to demonstrate the action of nicotine on the augmentors.

1. *Journ. Physiol.*, Vol. XIX, 1895.

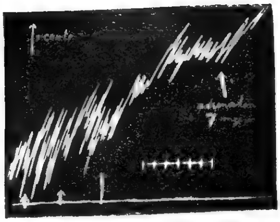

Fig. 5.—Non-pregnant uterus of cat. Cocaine hydrochloride mgms. 1, 2 and 4 given respectively at the first three arrows. Adrenalin given at the fourth arrow produced contraction of the uterus. Time = minutes.

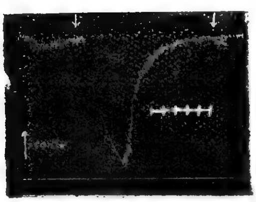

Fig. 6.—Non-pregnant uterus of cat previously ergotised to paralyse the augmentor fibres. Nicotine (10 mgms.) given at the first arrow, and repeated at the second. This shows stimulation followed by paralysis of the inhibitor nerves. The lever moves down during relaxation of the uterus. Time = minutes.

Fig. 7.—Non-pregnant uterus of cat. Nicotine (10 mgms.) produced relaxation of the uterus followed by contraction. Time = minutes. The lever passed below the level of the drum during relaxation of the uterus.

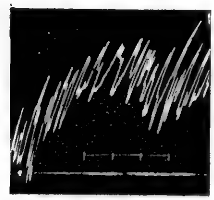

Fig. 8.—Uterus of cat. Action of ergotoxin (4 mgms.). Time = minutes.

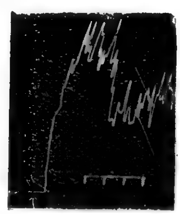

Fig. 9.—Uterus of guinea pig. Action of $BaCl_2$ (8 mgms.). Previous to administration of this drug no spontaneous movements were present.

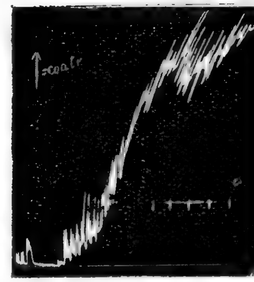

Fig. 10.—Uterus of rabbit, containing six ova. Action of tinct. strophanthus (0·6 c.c.). Time = minutes. Upstroke = contraction.

Fig. 11.—Uterus of rabbit. Action of quinine hydrochloride (2 mgms.). Time = minutes.

The action of nicotine on the uterus, in which both sets of nerves are responsive, is more difficult to explain. To a certain extent it depends, like that of adrenalin, on the predominant set. Fig. 7 represents the result obtained with a non-pregnant uterus of a cat, in which adrenalin produced decided relaxation. It will be noticed that nicotine at first produced relaxation as in the ergotised uterus, and this was followed, not by a gradual recovery, as in the former experiment, but by a sharp contraction. The spontaneous movements then recommenced at a higher tonus.

In another experiment (Fig. 4) on a non-pregnant cat, the action of adrenalin was first determined. The relaxation of the uterus which it produced was not great, probably because the muscle was nearly fully relaxed at the time the adrenalin was given. When the spontaneous movements returned 10 mgms. of nicotine were given, and produced relaxation equal in extent to that produced by adrenalin, but lasting only a short time. A strong contraction of the uterus then followed, succeeded by spontaneous movements at a relatively high tonus. The initial relaxation of the uterus is not present in all non-pregnant cats.

In the pregnant uterus of cats, and in the rabbit's uterus, in both of which the augmentory fibres predominate, and in the non-pregnant uterus of some cats, nicotine produces an initial contraction followed by exaggerated spontaneous movements at a high tonus. Relaxation is not produced by nicotine in these uteri.

Apocodeine prevents the stimulant action of nicotine on the uterus, in doses not sufficient to destroy the action of adrenalin.

I have already referred to the power of nicotine to reverse the action of adrenalin from inhibition to augmentation of the uterine movements. I have shown also that this property of nicotine cannot be explained on the ground that it paralyses inhibitory nerve cells, but that it is due to some more peripheral change in the relation of augmentory nerve endings to the muscle by which the latter is rendered more responsive to augmentory impulses. I have shown, also, that in those uteri in which the inhibitory nerves predominate, the nerve-cell excitation produced by nicotine, which at first results

in relaxation of the muscle, later produces contraction, and that finally the spontaneous movements continue at a higher tonus than formerly. I believe that this additional action of nicotine, in virtue of which it increases the irritability of augmentory nerve endings, accounts for all the three effects just enumerated, since none of them occur after ergotoxine, which paralyses these nerve endings.

It has been stated that nicotine stimulates plain muscle directly. Wertheimer and Colas attempted to show that nicotine stimulated the heart muscle directly, because it quickened the beat in an atropinised animal from which the cardiac sympathetic ganglia had been removed; Dixon[1] has shown that this effect is not produced if apocodeine be previously injected.

These experiments appear to show that nicotine acts on peripheral nerve cells: it stimulates both augmentors and inhibitors, and the stimulation is followed by depression of the nerve cells. When the inhibitory fibres predominate relaxation of the uterus is produced, but is followed by contraction and continuation of the movements at a higher tonus. It is clear that a comparatively small cause can alter the relative influence of the augmentor and inhibitory nerve fibres in the uterus. Pregnancy increases the influence of the augmentors, and nicotine exerts a like effect. Since nicotine does not paralyse the inhibitory nerve endings, it can only be supposed that it produces this effect by increasing the irritability of the augmentor nerve endings or of the muscle; the evidence is in favour of the former. This additional action of nicotine is on nerve endings because it influences the action of adrenalin.

Apocodeine prevents the initial stimulant action of nicotine on the uterine nerve cells. The spontaneous movements of the uterus can be revived by adrenalin after partial suppression by apocodeine.

Atropine antagonises the action of pilocarpine on the uterus, as on all other viscera, but it does not prevent the action of nicotine and adrenalin. Langley and Anderson have shown that atropine does not interfere with the results of electrical stimulation of the sympathetic to the uterus.

1. *Journ. Physiol.*, Vol. XXX, p. 105, 1903.

Given alone, atropine sometimes raises the tonus of the uterus. This effect is seen also when atropine is given after colchicine. (Fig. 3.)

It is thus evident that if atropine acts on nerve endings in the uterus, this action must be very slight, and is chiefly exerted on that portion of them upon which pilocarpine acts.

Drugs Acting on Plain Muscle Directly

Those drugs which act on plain muscle fibre directly naturally form an important group when dealing with such a muscular organ as the uterus. Many of the so-called abortifacients are included among these drugs. They act alike on all plain muscle fibre throughout the body, and are regarded as muscle poisons.

Those which in moderate doses stimulate the muscle are :—Strophanthus, squills, digitalis, apocynum, barium, lead, ergot, and quinine. Others destroy the movements. These are alcohol, chloroform, chloral, magnesium sulphate, hydrocyanic acid, and nitrites. The characteristic effect of stimulant muscle poisons is, besides strengthening the spontaneous contractions, to cause a considerable rise of tonus. The effect is better maintained than similar ones produced by drugs acting on the nervous mechanisms. Four milligrammes of ergotoxine (Fig. 8) maintained an increased peristalsis and tonus for three-quarters of an hour before the effect began to subside. Twenty-four milligrammes of barium chloride (Fig. 9) will continue its effect for several hours, and ultimately cause the death of the muscle. Strophanthus produces an equally powerful effect. (Fig. 10.) Quinine, although causing a great initial increase of tonus, does not maintain it so well. (Fig. 11.) Squills soon begins to destroy the spontaneous movements while maintaining the increased tonus. Digitalis is much less powerful. The tincture[1] of digitalis in this respect appears to have only one-tenth of the toxicity of the tinctures of strophanthus or squills. Apocynum produces a rise of tonus only. The spontaneous movements are destroyed by small doses of this drug.

Lead produced no results in my earlier experiments. In these

1. When the tinctures have been used, the alcohol has always been previously removed by evaporation.

the peritoneal covering of the uterus was undivided, and the muscle not directly exposed to the perfusion fluid, and was, no doubt, protected by an impervious coat of lead albuminate. Later, I divided the uterine wall so as to permit access of the fluid to the muscle fibres and into the interior of the uterus. I then obtained a powerful rise of tonus with lead, with death of the muscle in systole.

On increasing the doses of these drugs above that which produces the most beneficial results, the spontaneous movements begin to disappear, and finally the muscle dies in systole. Small doses of apocynum are sufficient to produce diminution of these movements; 0·2 c.c. of the tincture of apocynum produced a similar result to 0·4 c.c. of tincture of squills and 4 c.c. of tincture of digitalis. Apocynum, it should be remembered, is perhaps the most irritant poisonous member of the digitalis group of drugs—at least so far as its action on general tissues is concerned.

The results obtained with the digitalis group are comparable with their recognised action on the heart.

The muscle depressants need only a brief allusion. They are important chiefly as they include certain anaesthetics and narcotics used in midwifery.

Chloroform.—It was found that in animals anaesthetised by chloroform until the corneal reflex was abolished and then killed by bleeding, the uterus was just as active on removal as that from a pithed animal.

If the animal was killed by chloroform, and especially if this was done rapidly by a large initial dose, the spontaneous movements of the uterus were entirely suppressed, and the muscle fibres were fully relaxed; 0·05 per cent. of chloroform in the perfusion fluid arrested the uterine movements and lowered the tone of the muscle.

Chloral also relaxed the uterine muscle in much the same way.

Alcohol.—Drugs administered in alcoholic solution never produced satisfactory results.

One cubic centimetre of absolute alcohol added to the perfusion fluid, making a 0·3 or 0·4 per cent. solution, arrested the movements of the uterus, and produced relaxation of the muscle,

Hydrocyanic acid and magnesium sulphate at once destroy the uterine movements. Amyl nitrite produces a temporary depression.

The muscle poisons are more powerful in their action on the uterus than those drugs which act on the nervous mechanism. The direct muscle stimulants produce, as the principal feature of their action, a large and well-maintained rise of tone. The digitalis group are all uterine stimulants; strophanthus in this respect comparing favourably with ergot. Barium also has a powerful action on the uterus.

Conclusion

It will be observed from these experiments that the reaction of the uterus to drugs is compatible with its nature as a plain muscular organ supplied by the sympathetic nerve.

It must be remembered that both utero-inhibitory and utero-augmentory fibres are supplied by the sympathetic system, and both may be stimulated by the same drug. The transformation of the uterus during pregnancy may alter the relative influence of the two sets of fibres; nicotine appears to produce a similar change in the nervous mechanism.

I have great pleasure in acknowledging my indebtedness to Dr. W. E. Dixon for many valuable suggestions.

THE EFFECT OF ACID AND ALKALI ON THE OSMOTIC PRESSURE OF SERUM PROTEINS[1]

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The effect of alterations in the state of aggregation of colloids is of great importance in connection with the exchange of water in living cells. Many conditions affect the osmotic pressure of colloidal solutions, and such changes may be partially responsible for plasmolysis and turgor in plant cells and for the laking of red blood corpuscles.[2]

The relation of proteins to the exchange of water between blood vessels and lymph spaces was pointed out by Starling,[3] who was the first to measure the osmotic pressure of the proteins of blood serum. Moore and Parker,[4] whilst confirming the experimental results obtained by Starling in connection with the measurement of osmotic pressure of blood serum, criticised the suggestion that the endosmotic action of proteins could account for absorption of salt solutions from the connective tissue spaces into the blood vessels.

More recently Moore and Roaf[5] have discussed the question, whether the observed pressure readings are due to the electrolytes present in the solution. They point out that, although Reid,[6] by repeated precipitation and re-solution, obtains protein solutions which do not give evidence of osmotic pressure, his results are probably caused by a change in the 'solution aggregate'[7] of the protein.

Lillie,[8] using gelatin and egg-albumin, has brought forward

1. The expenses of this research have been partially defrayed from a grant given by the Government Grant Committee of the Royal Society.

2. cf. Lillie, *Amer. Journ. Physiol.*, Vol. XX, p. 167, 1907.
3. *Journ. Physiol.*, Vol. XIX, p. 312, 1896.
4. *Amer. Journ. Physiol.*, Vol. VII, p. 261, 1902.
5. *Bio-Chem. Journ.*, Vol. II, p. 34, 1906.
6. *Journ. Physiol.*, Vol. XXXI, p. 438, 1904.
7. Moore and Parker, *loc. cit.*, p. 262.
8. *Amer. Journ. Physiol.*, Vol. XX, p, 127, 1907.

evidence which confirms and amplifies the results of Moore and Roaf. He finds that proteins give an osmotic pressure when contained inside collodium sacs, whilst the crystalloids pass through easily not affecting the final pressure recorded. Further, non-electrolytes have practically no effect on the pressure, but electrolytes markedly influence the state of aggregation thus changing the osmotic pressure.

Although his experiments and reasoning are along similar lines to those of Moore and Roaf he is unfortunately misled as to their idea of the nature of the action of electrolytes on the osmotic pressure of the colloids. He states that they consider that the electrolytes 'in some unexplained manner confer on the colloid the power of exerting osmotic pressure.' But it is evident from their paper that they consider the effect as being due to a change in solution aggregate of the protein, although they do not attempt to explain the mechanism by which the electrolyte produces this change in the protein.

The two papers are so fully in accordance on certain fundamental deductions that it is desirable to state briefly the general conclusions given in both.

(i) The existence of a homogeneous solution and diffusion is evidence of osmotic pressure influences even if the pressure is too low for measurement by any method known at the present time.

(ii) The osmotic pressure is due to colloid, as all crystalloids could pass through the membranes used and would not give a permanent pressure.

(iii) There is no sharp line of demarcation between crystalloids and colloids but that they gradually merge into each other at the margins of the groups.

(iv) Electrolytes have a marked influence on the osmotic pressure of proteins.

The experimental work to follow was commenced in order to investigate further the effect of acid and alkali on the osmotic pressure of serum proteins.

Methods

The form of osmometer and the technique for filling it was the same as that previously described.[1]

The blood serum was obtained by centrifuging stirred pig's blood. A known quantity of standardised acid or alkali was taken and made up to the required volume by means of the serum. The *amount* of acid or alkali could then be expressed in terms of normal solutions.

These solutions were then immediately placed in the osmometer, and readings taken until a permanent osmotic pressure was recorded.

At the end of the experiment a known weight of solution was evaporated to dryness at 100 C. in a tared vessel, cooled in a desiccator, and weighed ; the residue was incinerated, and weighed again after cooling. In this way the weights of the solution, the total solids and ash were determined. By subtracting the weight of ash from the total solids the amount of organic matter was obtained and calculated as percentage of the weight of solution taken. The organic matter on the saline side of the membrane showed the amount of diffusible organic matter, and the percentage of this should be uniform throughout the solution ; therefore, the difference between the percentages of organic matter on the serum side and on the saline side gives the percentage of organic matter unable to pass through the parchment paper, and this amount is considered to be protein in nature.

The fluid used on the outer side of the osmometer was 0·9 per cent. sodium chloride to which an amount of acid or alkali had been added to make an equal strength to that in the serum placed on the other side of the parchment paper.

There was no heating of the serum with the alkali or acid added. Hence there was no hydrolysis directly into alkali or acid albumin such as would break up the protein molecule.

The osmotic pressure was finally expressed in millimetres of mercury for each 1 per cent. of protein present.

1. Moore and Roaf, *loc. cit.*, pp. 46-51.

Experiment I.—Pig's serum against 0·9 per cent. sodium chloride.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
16	...	3
40	...	7
64	...	9
88	...	14
112	...	21
136	...	25
160	...	25
184	...	25
208	...	25

	On serum side	On saline side
Percentage of organic matter	6·762	0·136
Percentage of protein by difference	6·626	

Osmotic pressure for each 1 per cent. of protein = 3·7 mm. Hg.

Series I.—Experiments in which Alkali was Added to Serum

Experiment II.—0·25 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0·025 N, against 0·9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
17	...	21
41	...	27
65	...	27
89	...	27
114	...	27
138	...	27
162	...	27
185	...	27

	On serum side	On saline side
Percentage of organic matter	4·619	0·157
Percentage of protein by difference	4·462	

Osmotic pressure for each 1 per cent. of protein = 6·05 mm. Hg.

Experiment III.—0·375 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0·0375 N.; against 0·9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
14	...	24
39	...	30
62	...	33
86	...	35
110	...	36
134	...	37
158	...	37
183	...	37
208	...	37

	On serum side	On saline side
Percentage of organic matter	5·772	0·359
Percentage of protein by difference	5·413	

Osmotic pressure for each 1 per cent. of protein = 6·83 mm. Hg.

Experiment IV.—0·5 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0·05 N, against 0·9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
15	...	30
38	...	58
62	...	66
86	...	70
110	...	70
134	...	70

	On serum side	On saline side
Percentage of organic matter	9·294	0·390
Percentage of protein by difference	8·904	

Osmotic pressure for each 1 per cent. of protein = 7·86 mm. Hg.

Experiment V.—0·75 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0·075 N, against 0·9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
16	...	34
40	...	60
65	...	64
88	...	64
112	...	65
136	...	65
160	...	65
184	...	65

	On serum side	On saline side
Percentage of organic matter	7·009	0·261
Percentage of protein by difference	6·748	

Osmotic pressure for each 1 per cent. of protein = 9·63 mm. Hg.

Experiment VI.—1·25 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0·125 N, against 0·9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
13	...	34
40	...	63
61	...	68
85	...	73
109	...	78
133	...	80
157	...	83
181	...	86
207	...	91
229	...	95
253	...	95
277	...	95

	On serum side	On saline side
Percentage of organic matter	8·308	0·325
Percentage of protein by difference	7·983	

Osmotic pressure for each 1 per cent. of protein = 11·89 mm. Hg.

Experiment VII.—2·5 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, ~~making a~~ strength of alkali equivalent to 0·250 N, against 0·9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
12	...	31
15	...	41
37	...	79
60	...	97
84	...	107
108	...	115
132	...	121
160	...	129
184	...	130
208	...	130

	On serum side	On saline side
Percentage of organic matter	7·218	0·213
Percentage of protein by difference	7·005	

Osmotic pressure for each 1 per cent. of protein = 18·54 mm. Hg.

Experiment VIII.—3·75 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0·375 N, against 0·9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
15	...	57
39	...	78
63	...	104
88	...	123
111	...	128
135	...	132
159	...	134
183	...	135
207	...	136
231	...	136

	On serum side	On saline side
Percentage of organic matter	6·262	0·511
Percentage of protein by difference	5·751	

Osmotic pressure for each 1 per cent. of protein = 23·65 mm. Hg.

Experiment IX.—5 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0·500 N, against 0·9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
13	...	28
37	...	54
61	...	86
85	...	97
109	...	97
133	...	91
157	...	87
182	...	85
206	...	81
229	...	75

	On serum side	On saline side
Percentage of organic matter	4·851	1·502
Percentage of protein by difference	3·349	

Osmotic pressure for each 1 per cent. of protein = 22·39 mm. Hg.

Experiment X.—7·5 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0·750 N, against 0·9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
15	...	27
39	...	45
63	...	67
87	...	78
111	...	74
135	...	72
159	...	71

	On serum side	On saline side
Percentage of organic matter	6·319	1·825
Percentage of protein by difference	4·494	

Osmotic pressure for each 1 per cent. of protein = 15·76 mm. Hg.

In this series of experiments it is seen that the osmotic pressure for each 1 per cent. of protein is higher the greater the amount of alkali added until a maximum is reached. Above this maximum the osmotic pressure diminishes because the alkali acts on the protein splitting it up into products that can pass through the parchment paper. Thus in Experiments IX and X no permanent pressure is produced, and after the pressure reaches a maximum it slowly falls. That this fall is due to dyalysis of protein decomposition products is shown by the increased percentage of organic matter in the fluid on the saline side of the osmometer.

Series II.—Experiments in which Acid was Added to Serum

Experiment XI.—0·5 c.c. of 14·6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0·02 N, against 0·9 per cent. sodium chloride containing the same strength of acid.

This experiment was continued for a period of 264 hours during which time no evidence of osmotic pressure was noticed. At the end of the experiment no leak could be found in the apparatus.

Experiment XII.—1·0 c.c. of 14·6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0·04 N, against 0·9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
17	...	0
41	...	0
65	...	0
89	...	0
113	...	1
137	...	2
161	...	3
185	...	4
209	...	5
233	...	5
257	...	5

	On serum side	On saline side
Percentage of organic matter	6·743	0·162
Percentage of protein by difference	6·581	

Osmotic pressure for each 1 per cent. of protein = 0·76 mm. Hg.

Experiment XIII.—1·5 c.c. of 14·6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0·06 N, against 0·9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
16	...	0
40	...	9
64	...	12
88	...	18
112	...	19
136	...	24
160	...	24
184	...	24
209	...	25
232	...	25
256	...	25

	On serum side	On saline side
Percentage of organic matter	6·716	0·324
Percentage of protein by difference	6·392	

Osmotic pressure for each 1 per cent. of protein = 3·91 mm. Hg.

Experiment XIV.—2·0 c.c. of 14·6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0·08 N, against 0·9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
14	...	0
38	...	1
62	...	2
86	...	3
110	...	10
134	...	15
158	...	17
182	...	18
207	...	19
230	...	23
254	...	24
278	...	29
302	...	32
326	...	32

	On serum side	On saline side
Percentage of organic matter	5·924	0·485
Percentage of protein by difference	5·439	

Osmotic pressure for each 1 per cent. of protein = 5·87 mm. Hg.

Experiment XV.—3·0 c.c. of 14·6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0·12 N, against 0·9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
15	...	32
43	...	42
63	...	43
87	...	43
111	...	42
135	...	43
159	...	43

	On serum side	On saline side
Percentage of organic matter	6·052	0·400
Percentage of protein by difference	5·652	

Osmotic pressure for each 1 per cent. of protein = 7·61 mm. Hg.

Experiment XVI.—3·5 c.c. of 14·6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0·14 N, against 0·9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
15	...	0
39	...	4
63	...	9
87	...	9
111	...	10
135	...	14
159	...	16
183	...	17
207	...	17

	On serum side	On saline side
Percentage of organic matter	6·755	0·565
Percentage of protein by difference	6·190	

Osmotic pressure for each 1 per cent. of protein = 2·75 mm. Hg.

Experiment XVII—4·0 c.c. of 14·6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0·16 N, against 0·9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
15	...	0
39	...	0
63	...	0
87	...	1
111	...	7
135	...	13
159	...	18
183	...	18

	On serum side	On saline side
Percentage of organic matter	7·612	0·276
Percentage of protein by difference	7·336	

Osmotic pressure for each 1 per cent. of protein = 2·45 mm. Hg.

Experiment XVIII.—4·5 c.c. of 14·6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0·18 N, against 0·9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
16	...	0
40	...	0
64	...	0
88	...	0
112	...	1
136	...	2
160	...	4
184	...	8
208	...	12
232	...	16
256	...	18
280	...	20
304	...	22
328	...	23
352	...	24
376	...	25
400	...	25
424	...	25

	On serum side	On saline side
Percentage of organic matter	6·830	0·581
Percentage of protein by difference	6·249	

Osmotic pressure for each 1 per cent. of protein = 4·00 mm. Hg.

In these experiments it is seen that the addition of a small amount of acid causes the osmotic pressure of serum proteins to fall so that only zero readings can be obtained. This amount of acid (0·02 N) is very nearly the same as that found by Moore and Wilson for the ash of serum (0·03 N)[1] using 'di-methyl' as an indicator. Further addition of acid causes a rise of pressure, but beyond a certain strength of acid the pressure drops and becomes irregular. This drop is not due to the acid splitting up the protein into hydrolytic products capable of passing through the membrane because there is no corresponding rise in the total solids of the outer fluids, such as was seen in the experiments with the stronger strengths of alkali. The solutions which gave the varying pressures showed a yellow flocculent precipitate, and the fall of pressure was therefore probably due to partial precipitation of the protein from the solution.

1. *Bio-Chem. Journ.*, Vol. I, p. 297, 1906.

The results obtained in the experiments are summarised in the following Table.

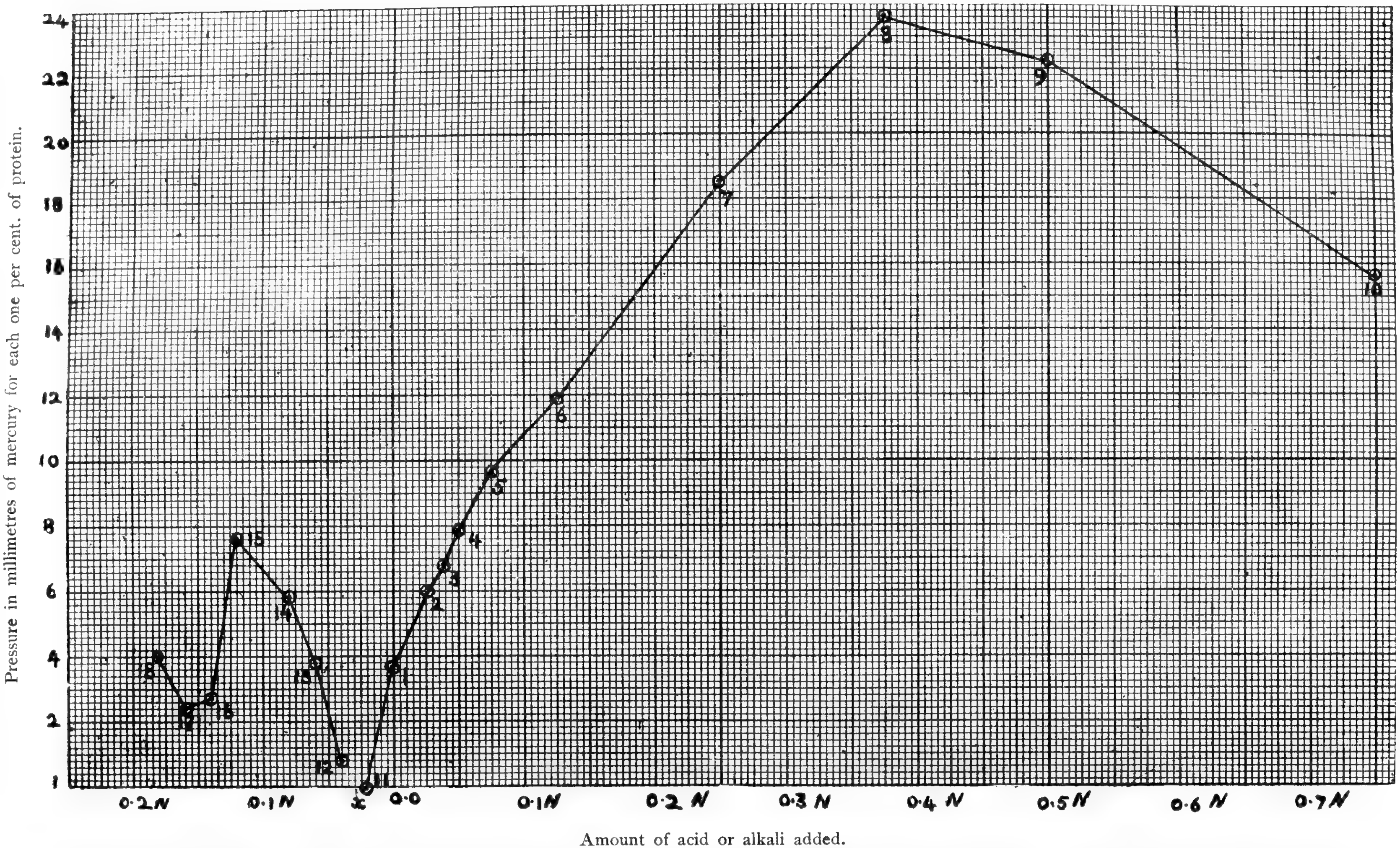
ALKALI ADDED

No. of Experiment	I	II	III	IV	V	VI	VII	VIII	IX	X
Alkali added to serum expressed in normal concentrations ...	0	0·025	0·0375	0·05	0·075	0·125	0·25	0·375	0·50	0·75
Corresponding osmotic pressure in mm. of mercury per 1 per cent. of protein present ...	3·7	6·05	6·83	7·86	9·63	11·89	18·54	23·65	22·39	15·76
Value of solution aggregate corresponding to the osmotic pressure observed	45,887	28,063	24,859	21,601	17,631	14,279	9,157	7,179	7,583	10,773

ACID ADDED

No. of Experiment	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
Acid added to serum expressed in normal concentrations ...	0·02	0·04	0·06	0·08	0·12	0·14	0·16	0·18
Corresponding osmotic pressure in mm. of mercury per 1 per cent. of protein present ...	0	0·76	3·91	5·87	7·61	2·75	2·45	4·00
Value of solution aggregate corresponding to the osmotic pressure observed	0	223,400	43,423	28,924	22,310	61,739	69,300	42,446

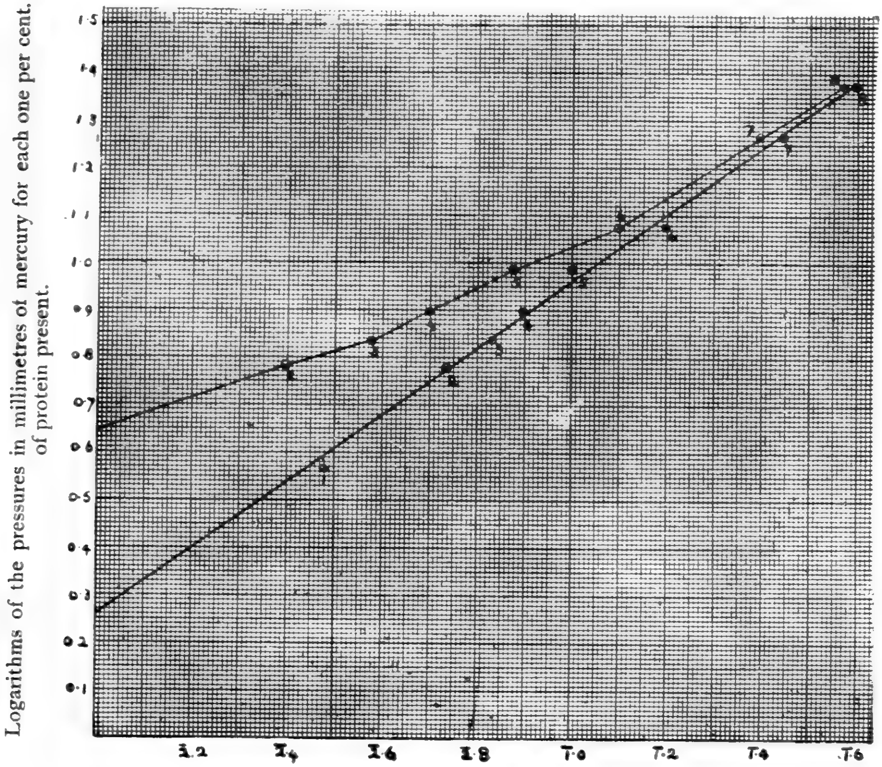
These results are plotted together in the accompanying curve (Fig. 1) where the ordinates represent pressures in millimetres of mercury for each one per cent. of protein, and the abscissae show the amount of normal acid or alkali added to the serum. The zero point marks the pressure obtained with normal serum, whilst x shows the point of neutrality of the ash of serum as obtained by Moore and Wilson. If the point x be considered as the true neutral point then the alkalinities should be corrected by adding this amount (0·03 N) to the actual quantity of alkali added, whilst the acidities should have the same amount subtracted. The value of this correction is strikingly shown in the figure, where the continuation of the acid curve practically cuts the zero pressure at the point x. The two following curves (Fig. 2) show the effect of plotting the logarithms of the amounts of alkali against the logarithms of the pressures. The upper curve is plotted using the amount of alkali actually added to the serum, whilst the lower figures are corrected for the amount of alkali present in the ash of serum. The lower curve shows a logarithmic relation between the two sets of figures, so that the neutral point of serum ash determined by Moore and Wilson must have some



o·o shows the normal serum. The amount of acid added reads to the left, and the amount of alkali added reads to the right; x shows the neutral point of serum ash as obtained by Moore and Wilson (0·03 N). The numbers on the curve show the number of the experiment from which the point was determined.

relation to the influence of reaction on the osmotic pressure of serum proteins.

Apparently the point of exact neutrality corresponds to the condition in which the osmotic pressure is least. This would explain many points in connection with determinations of osmotic pressure of proteins. Reid[1] by repeated precipitation and washing removed the



Logarithms of the amounts of normal alkali present. Upper curve shows results obtained experimentally; lower curve shows results when the amount of alkali found by Moore and Wilson for serum ash is added to that actually used. The numbers on the curves show the number of the experiment from which point was determined.

alkali of the serum, and the partial return of osmotic pressure that he obtained on adding some of the washings to the pressure-free protein could be due to a little of the alkali in the washings acting in the same way as the alkali in the present series of experiments. Lillie[2] found

1. *Loc. cit.*
2. *Loc. cit.*, p. 138.

that addition of a small quantity of acid caused a marked drop of osmotic pressure, and that a little more acid caused the pressure to rise again. In the case of egg-albumin this rise was never up to the original pressure for the normal egg-albumin, but with gelatine the pressure with larger quantities of acid rose much above its original value.

The osmotic pressure of blood serum obtained by Starling was slightly greater than that obtained by Moore and Parker or Moore and Roaf. This would be due to Starling using the protein-free filtrate of serum as the solution against which the osmotic pressure was determined whilst the other authors used salt solution, and the amount of alkali would be greater in the former case than in the latter. It is interesting to note that in precipitating proteins by neutral salts it is usual to add a trace of acid in order to obtain more complete precipitation. This would neutralise the slight degree of alkalinity usually associated with natural proteins and bring about the condition where there is least osmotic pressure, and hence the colloid being in a less stable condition it is more easily precipitated.

The cause of the change in 'solution aggregate' brought about by the change in reaction has not been determined. In the case of the alkaline solutions it is not due to an ordinary hydrolysis of the protein. Hydrolytic action would be progressive, and should cause a gradual increase of diffusible organic matter as the strength of alkali is raised; but there is no increase until the alkali reaches 0·37 normal, when hydrolytic products appear in the saline solution on the outer side of the osmometer. Lillie[1] found that the similar rise of osmotic pressure produced by treating gelatin with acid, disappears slowly on removal of the acid by dialysis. If the change were due to slight hydrolysis, with weaker strengths of alkali one must assume that the amphoteric electrolytes produced neutralise the alkali so that the change does not proceed beyond an early stage and that removal of the alkali allows the protein to become re-associated into its original state. The increase in colloidal particles is unlikely to be due to the alkali lowering the surface tension between the solvent and solute, because addition of

1. *Loc. cit.*, p. 142.

inorganic salts usually raises the surface tension at an air-water surface.

The action of acid is probably of the same nature as that of alkali but owing to the onset of partial precipitation the series of experimental determinations is not so extensive. This partial precipitation points to some change which overcomes the tendency of the osmotic pressure to increase and which allows some constituent of the serum to form solution aggregates of increased size until the aggregates become of such size that they are visible to the naked eye.

The relationship of these changes in solution aggregate (osmotic pressure) to the action of neutral salts in effecting plasmolysis and laking, and to the effect of acid and alkali on cell growth and cell division, is being further studied.[1]

Conclusions

1. Alkali causes an increase in the osmotic pressure of the colloidal constituents (proteins) of blood serum. This increase is not directly proportional to the amount of alkali added.

2. Acid causes the osmotic pressure of the colloidal constituents (proteins) of blood serum to fall to zero when small amounts of acid are used, but with larger quantities the pressure rises again.

3. The point at which zero pressure exists practically corresponds to the alkaline 'reactivity' of serum ash, obtained by Moore and Wilson, using di-methyl-amido-azo-benzole as an indicator.

4. When the amount of alkali added is corrected by the above-mentioned alkaline 'reactivity' the relationship of the osmotic pressure to the amount of alkali is a logarithmic one. Similarly corrected, the relationship of the increase of osmotic pressure on addition of acid is probably logarithmic to the amount of acid added, but the series of experimental observations is not long enough to give an accurate curve. These facts confirm the accuracy of the observations of Moore and Wilson in determining the reaction of blood serum ash.

1. For a comparison with the action of various agents on the swelling of gelatine (which probably depends on colloidal osmotic pressure) see W. Pauli, *Ergebnisse der Physiologie*, Vol. VI, p. 105, 1907.

5. The maximum of pressure obtained by adding alkali is limited by the breaking up of the protein into particles small enough to pass through the parchment paper membrane, whilst the maximum pressure on the addition of acid is reached when the protein is partially precipitated from solution.

In conclusion we wish to thank Professor Benjamin Moore for much help during the course of this research.

UROBILIN EXCRETION IN DISEASED CONDITION

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Various theories have been propounded to explain the source of urobilin in urine. In health it is believed to be derived from bilirubin by the action of bacteria present in the alimentary canal. In vitro this change has been accomplished successfully; but the point has not been settled whether one special microbe does it or whether there are several varieties capable of effecting the change. Again, urine containing a large amount of bile does not show any urobilin band when inoculated with faecal microbes, so the production of urobilin from bile-pigments by the action of faecal microbes is not possible under these conditions. The same experiment performed with ox-gall broth and similar germs, neither gives the urobilin band nor can urobilin be extracted with amyl alcohol from the mixture. Bacterial action is surmised to be responsible for the production of urobilin on account of the fact that meconium is rich in bile and urobilin is absent, while it appears soon after birth. However, various changes take place in the alimentary canal of the new-born besides the appearance of the bacteria. Thus the different glands of the canal are called into activity by the presence of the milk in it. For this reason it is quite as logical to attribute the presence of urobilin to the action of the secretion of these glands on bile-pigments as to the presence of bacteria. Vaughan Harley (1) found that urobilin in faeces was increased along with the aromatic sulphates in the urine of certain dogs.

This seems to show that bacterial agency may be responsible for the increase of urobilin as the same is true for the increase of aromatic sulphates. The following observations, unfortunately, do

not show any such constant relationship between increase of urobilin and indican in urine.

Thus in 124 cases where urobilin was found to be copious, only 54 (*i.e.*, 43·2 per cent. of cases) had copious indican also, while the rest had traces.

On the other hand out of 126 cases with copious indican only 53 (*i.e.*, 42·0 per cent.) had copious urobilin also. Thus, whether we look for increased indican in cases of copious urobilin or *vice versa*, the fact remains that in diseased condition at least in more than 50 per cent. of cases the reverse happens to be true.

Again, out of 188 cases with traces of urobilin 67 (35·5 per cent.) had copious indican; while out of 157 cases of traces of indican 53 (*i.e.*, 33·8 per cent.) had increased urobilin. These observations show a higher percentage in favour of the correlation theory of Vaughan Harley. However, we must take into consideration this fact that both these constituents are found in traces under ordinary circumstances in the urine, consequently in those cases where either of the substances is scanty the other ingredient may be scanty also, although the cause of the production may not be the same.

Again, in a case of chronic constipation where one finds copious indican, according to the views of Vaughan Harley one ought to find copious urobilin also; however, in such a case urobilin was actually found in traces only.

On the other hand in a case of chronic diarrhoea indican was found in traces while urobilin was copious and free. In a case of phthisis where the urine was repeatedly examined to see if any relationship was to be noticed between these two constituents of urine, indican was constantly found to be in traces while urobilin was copious and free, except on two occasions when the patient had indigestion followed by diarrhoea, when indican was found to be moderately increased while urobilin was copious as usual. In a case of puerperal septicaemia although indican was copious, urobilin was found to be in traces only. In a case of enteric fever urobilin was found to have increased considerably although indican was found in traces. The

same was true in a case of Gaertner infection during convalescence. On the other hand in two cases of coli infection urobilin was in traces although indican was increased.

Thus, whether one takes the statistics of several cases or looks to individual cases where indican is in excess, it is evident that no definite relationship exists between urobilin and indican present in urine.

In these observations urobilin was extracted with 10 c.c. amyl alcohol from 30 c.c. urine. The spectrum of pure urine as well as of the extract was examined, and those cases where a deep band was seen between the green and blue parts were considered as having copious urobilin. In many of these cases the band was found in pure urine. Alcoholic solution of zinc chloride and ammonia was subsequently added to the amylic extract, and the amount of fluorescence noted as control. Indican was tested by mixing 15 c.c. urine with equal parts of pure hydrochloric acid, and to this one drop of either fresh hydrogen peroxide or 0·5 per cent. solution of nitrite of sodium was added and the colour of the chloroform extract was noted for the amount of indican. Some specimens of urine do not require the oxidising agent at all—rather the reverse.

Urobilin was also looked for in various diseases, with a view to determine whether the increase is due to any particular disease or otherwise, so that this fact may be used for differential diagnosis. These observations agree with those of others in several respects; however, in some cases this does not hold true.

In enteric fever it has been generally stated that urobilin is not increased. In twenty-one cases of this disease only three had increased urobilin, but two of these cases had complications which must have been responsible for the increase, while the third had increased urobilin during convalescence only. In a case of enteric the patient developed croupous pneumonia as a complication; still no increase of urobilin was noticed for the succeeding five days, after which it showed a good deal. No complication of the liver had arisen during this period. In a case where the patient's blood did not respond to the Widal test for *B. typhoid* but to that of *B. gaertner*, urobilin was found

increased during convalescence; and in two other cases where agglutination was obtained with *B. coli*, urobilin was found scanty. In a case where the blood clumped both *B. coli* and *B. gaertner*, urobilin was scanty. Finally a case which did not clump any bacilli of the typho-coli group but clumped a bacillus isolated from the patient's urine, showed only traces of urobilin on several occasions. This microbe belongs to the typho-coli group with certain special characters which will form the subject of another communication. In the three cases of Gaertner and coli infection it is interesting to state that indican was copious.

In diseases of the respiratory system it was found that both croupous and broncho-pneumonia had copious urobilin in all cases (seventeen); while asthma, pleurisy, and bronchitis (five cases of each disease) did not show any increase. In a case of croupous pneumonia, urobilin was found to be normal during the convalescent stage. In a case of tubercular pleurisy it was copious. Here again indican was in traces.

In phthisis (fifty-seven cases) it was found increased in 75·5 per cent. of cases. In very early cases it was not increased. Here it may be stated that in two out of three cases of leprosy it was found to have increased considerably. This increase of urobilin in phthisis is interesting because it has been observed that injection of tuberculin has a similar effect (2).

In diabetes it has been stated by Schäfer (3) that 'urobilin was increased as a rule.' In only six cases have I found it to be increased out of twenty-five cases of this disease.[1] One of the cases with increased urobilin died, and at the *post mortem* examination chronic tuberculosis was found to be present. The pancreas was not diseased. In another case the patient had a severe crop of boils at the time when urobilin was copious, and it was only in traces after recovery from this complication; the amount of sugar was 5 per cent.

It is curious that in a large number of cases of uric acid diathesis

1. It may be suggested that as in diabetes the amount of urine is considerably increased, the quantity used must be proportionately larger than that used for other diseases. With this object, in two cases 100 c.c. of such urine were extracted with 10 c.c. amyl alcohol. Urobilin was not found in sufficient quantity to give even a faint band.

urobilin has been found rarely increased. The samples examined were loaded with urates and were high coloured, yet this constituent was only found increased in five out of twenty-five cases. On the other hand indican was found to be copious in thirteen out of twenty-five cases of the same complaint. This also shows that in a large number of cases of increased intestinal putrefaction, urobilin is not necessarily increased.

In six cases of acute dysentery urobilin was scanty, and in four cases of sprue the same was true. Out of three cases of appendicitis only one had excess of urobilin, while a case of acute peritonitis had traces of urobilin. Again, eighteen cases of renal calculi and pyelitis had only traces of urobilin. Out of seven cases of acute rheumatism urobilin was found to be copious in six cases, while the seventh case had traces only. This last case had copious uric acid also, while the others had ordinary amount of uric acid, so it is likely that the disease was more of the nature of uratic deposit in the joints rather than rheumatic fever. However, it is not likely that uric acid prevents the excretion of urobilin, as in some cases of phthisis and other diseases both urobilin and uric acid were copious in the same sample of urine.

On the other hand in six cases of scurvy both urobilin and indican were copious. It seems that in these cases the destruction of the effused blood in the tissues is responsible for the urobilin, while the intestinal putrefaction was responsible for the indican.

It has been stated that in acute Bright's disease urobilin is not excreted by the kidneys. Out of twelve such cases three had copious urobilin, though the urine was loaded with casts, both hyaline and granular.

These observations show that urobilinuria can exist in cases of albumen with casts in the urine. Again from the records of other diseases where both albumen and urobilinuria were looked for, I find that in many other diseases also this holds true. Thus in case of phthisis out of twenty-five cases where albumen was found in traces, sixteen had copious urobilin and more had traces only. Besides that, in one case of phthisis with copious albumen (varying between three

and eight parts per 1,000), urobilin was also copious on every occasion that the urine was examined (thirteen samples examined).

In enteric fever as a rule urobilin is not increased ; however, three cases with copious urobilin had albumen also. In one of these cases perhaps the pus present in the urine might have been the cause of albumen. In malaria, out of four samples with albumen three had copious urobilin, one of these had granular casts also. Relapsing fever cases with albumen in the urine showed copious urobilin in three samples, while in two such cases the urobilin was in traces ; one of these latter had casts also. In eight cases of plague with albumen in urine, seven showed copious urobilin. Ten cases of pneumonia with albumen in the urine showed copious urobilin also. Two cases of acute metritis showed both albumen and urobilin in large amount. Out of four cases of heart disease with renal complication, copious urobilin was found in three samples.

In diabetes, urobilin was not found much increased as a rule ; however, in two cases both albumen and urobilin were in excess. In three cases of septic infection the same has been found true.

In a case of sunstroke, albumen was three parts per 1,000, casts were also present ; however, free urobilin was present in moderate amount, *i.e.*, a fairly well-marked band was present. A case of filariasis had copious urobilin in conjunction with albumen during the acute attack of orchitis and lymphangitis. Finally, in a case of malignant disease of the liver both albumen and copious urobilin were found to be present.

In fevers red blood corpuscles suffer most destruction, as in malaria (eighteen cases), relapsing fever (six cases), and plague (nine cases) urobilin was considerably increased—in fourteen, five and six cases respectively. In a case of kala-azar, and another of cerebro-spinal meningitis, urobilin was found in traces only. Both these cases had copious indican and uric acid. On the other hand hepatic affection did not show any increased urobilin as a rule. Thus, out of thirty-seven cases of hepatitis only thirteen had increased urobilin. Again, out of these, twenty-five cases had jaundice. Eleven of these cases had copious urobilin ; however, two had tubercle, so the increased urobilin may

be attributed to that complication. In most of the cases when the faeces was examined for the presence of urobilin (mercury perchloride), it was found to be absent when urobilin was absent in the urine also; however, in one case although no urobilin could be detected in the faeces it was found to be excessive in the urine. Surely in this case the source of the urobilin in the urinary secretion was not the alimentary canal. Both bile-pigments and salts were found in moderate amount in the urine of this case. In one case of jaundice the urine had only traces of urobilin during the commencement of the illness, when there was a good deal of bile-pigments and salts, while at a later period as the patient was getting better urobilin was found to be copious and at the same time bile salts and pigment were in traces only. Now at this stage the patient's skin and conjunctivae were still deeply jaundiced. What is the significance of this? Was this a case of simple jaundice to begin with and that it became one of urobilin jaundice at a later period, or that the bile-pigment in the tissues was gradually eliminated as urobilin without the help of the liver? The latter supposition is much more likely. It is stated by von Noorden (4) that urobilin as such is not present in the blood but as bile-pigment, and that it gets excreted in the urine as urobilin. If this be so, why is it that bile-pigment is excreted as such in most cases of jaundice? It may be suggested that as long as the bile ducts are blocked up, the bile-pigment in the tissues gets excreted as bile-pigment in the urine; but when the ducts become patent, the liver being relieved of the pent-up bile is able to re-absorb the bile from the tissues, whence it reaches the alimentary canal where it is converted into urobilin. This is the usual explanation; but is very roundabout, and presupposes the existence of urobilin in blood, which has not been found to be the case. Serum obtained from the blood of such cases is deep yellow coloured but does not show the spectrum of urobilin, although the urine may be full of it.

Again, in case of digestive trouble where indican is increased, and also in uric acid diathesis where the liver is generally at fault, urobilin has not been found to be increased generally, so it is much more probable that the bile-pigment in the blood gets changed into

urobilin somewhere else, perhaps in the kidneys (von Noorden (4), von Jaksch (5)).

Out of four cases of hepatic abscess (tropical) urobilin was found to be increased in one case only, and this was a case where the abscess had burst into the lung producing secondary pneumonia at the same time.

Out of eight cases of cirrhosis of the liver only two cases showed much urobilin. One of these cases had severe scurvy also, so one can attribute the presence of urobilin to the scorbutic condition rather than to the cirrhotic liver. Thus, at least in pathological conditions of the liver, this organ does not seem able to produce urobilinuria, while in the case of hepatic abscess with secondary pneumonia, and in cirrhosis of the liver complicated with scurvy, urobilin was in excess. Pneumonia and scurvy are diseases which have invariably shown the presence of copious urobilin in the urine.

In cases of malignant tumours of abdominal organs, urobilin was increased in five out of eleven cases. Seven of these cases had cancer of the liver and four of these had copious urobilin (von Noorden (6)).

In a case of malignant disease of the larynx, and another of the jaw, urobilin was not increased.

As regards nervous diseases it may be stated that in five cases of chronic myelitis, urobilin was found to be copious; while in three cases of peripheral neuritis it was in traces. Out of two cases of beri-beri, one showed excess of urobilin. In a case of hemiplegia due to haemorrhage urobilin was not increased even a few days after the attack. Probably the haemorrhage was not excessive. This case was interesting on account of the fact that both indican and uric acid were copious. A case of neuromata did not show any excess of urobilin. In the cases of chronic myelitis the onset was accompanied with severe fever, so the inflammation of the spinal cord must have been due to some specific infection. A case of pseudo-hypertrophic paralysis did not show any excess of urobilin.

In cases of septic infections it was not always that urobilin was found increased. Out of fourteen cases seven showed an excess.

Out of five cases of filariasis only one showed excess, and that was at a time when there was high fever with orchitis.

In some of the diseases mentioned above the observations have been made in such a small number of cases that it is not possible to try to generalise about the relation of urobilinuria to such conditions; however, these show that the liver and the intestinal putrefaction do not play any important part in the production of excess of urobilin, at least in diseased conditions.

Those diseases which produce the destruction of red blood cells seem to be much more prone to show urobilin in urine. It may be stated here that in three cases of pernicious anaemia urobilin was copious, an observation which agrees with that of others. Thus malaria, relapsing fever, plague, scurvy, and tuberculosis show increased urobilinuria, as compared with cases of uric acid diathesis, hepatic diseases, dysentery, sprue, and diabetes. All these conditions are often accompanied by digestive disturbance, and increased intestinal putrefaction, as is shown by the increased excretion of indican in such states.

As regards the question whether the increase of urobilin in urine helps the diagnosis of disease, it seems that although this condition is not pathognomonic of any one disease, it can help one at differential diagnosis of some conditions. Thus one often meets with cases of remittent fevers which clinically appear to belong to the group of the enteric type, but Widal test is not obtained with some of the typho-coli group of bacilli which are generally available in a laboratory on the one hand, while malarial parasites are absent on the other.

Thus in one of the cases mentioned above where the typho-coli bacilli did not give the reaction, it was afterwards found that a bacillus of this group isolated from the urine of the patient gave Widal test quite readily. Urobilin was found in traces on every occasion that the urine was tested in this case.

In such cases the diagnosis generally lies between malaria, enteric, and tuberculosis.

Ehrlich's diazo reaction if present in such a case will exclude malaria, especially so if the hyaline large mononuclear leucocytes

are not in large number. In regard to tuberculosis, Calmette's reaction and the opsonic index are supposed to be of no value in such a condition, as the ophthalmo reaction has been found in genuine cases of typhoid and, again, the opsonic index has been lowered for tubercle bacillus in similar cases.

In such a case an excessive amount of urobilin will increase the probability of tubercular infection being present.

As regards the question whether urobilin and indican excretion in urine show any relationship which warrants one in assuming that the originating cause of the former in diseased states is intestinal putrefaction, it would seem that it must be answered in the negative.

Again, these observations seem to indicate haemoglobin destruction to be the principal cause of urobilinuria, in diseased conditions at least, rather than derangement of hepatic functions.

In conclusion it may be stated that nearly 500 samples of urine were examined to determine the various facts mentioned in this paper.

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(1) *British Medical Journal*, Vol. II, p. 898, 1906.

(2) von Jaksch, *Clinical Diagnosis* (English Edition), 1905.

(3) Schäfer, *Text-Book of Physiology*, Vol. I, p. 629.

(4) von Noorden, *Metabolism and Practical Medicine* (English Edition), Vol. II, p. 214.

(5) von Jaksch, *Clinical Diagnosis* (English Edition), 1906.

(6) von Noorden, *Metabolism and Practical Medicine* (English Edition), Vol. III, p. 818.

VARIATIONS IN THE FREE HYDROCHLORIC ACID OF THE GASTRIC CONTENTS IN CANCER AND THE SO-CALLED 'PHYSIOLOGICALLY ACTIVE' HYDROCHLORIC ACID

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In a paper recently published in the *Proceedings of the Royal Society*,[1] Messrs. Copeman and Hake describe experiments carried out upon the acidity of the gastric contents in mice and rats with transplanted tumours, and also in a number of cases of cancer in man.

The authors state that their results contradict or materially modify the conclusions arrived at by Moore and others,[2] but I desire no better confirmation of our results than is given by the table of Messrs. Copeman and Hake when using our methods, which are those that have been established by the usage of many previous observers. Because somewhat different results have been given by another method intended to show the so-called 'physiologically active' hydrochloric acid, Copeman and Hake come to the conclusion that this quantity is not materially decreased in cancer in man, and is actually somewhat increased in mice with 'cancerous' growths.

For reasons which will be given later, I am of opinion that this term 'physiologically active hydrochloric acid' is a misnomer, and that the method used shows something quite different from hydrochloric acid in any active form. The method will be criticised after dealing with the other results obtained by Copeman and Hake, but

1. *Proc. Roy. Soc.*, Series B, Vol. LXXX, p. 444, 1908.

2. Moore, Alexander, Kelly and Roaf, *Proc. Roy. Soc.*, Series B, Vol. LXXVI, p. 138, 1905; *Bio-Chem. Journ.*, Vol. I, p. 274, 1906; Wilcox, *Lancet*, June 10, 1905, p. 1566; Morton Palmer, *Bio-Chem. Journ.*, Vol. I, p. 398, 1906; *Guy's Hospital Reports*, Vol. LX, p. 181, 1906.

before passing to this criticism I consider it essential to point out what was really shown regarding the variations in the gastric acidity in malignant disease and other abnormal conditions in the two papers by myself and collaborators, especially since Copeman and Hake have fallen into serious errors regarding our position and results.

Before the appearance of our first paper it was well known that the amount of hydrochloric acid in the stomach contents in cases of carcinoma *of the stomach* was as a general rule much reduced, and in many cases fell to a zero value, and it was equally well known that there were well-marked exceptions to this rule in which the hydrochloric acid was as high or higher than normal.

These facts are supported by a wealth of literature which need not be quoted here.

Our first paper demonstrated by a series of analyses carried out by several different methods, in a number of cases of *cancer in other situations than the stomach*, that exactly the same facts held for the acidity of gastric secretion, no matter where the malignant growth was situated.

The experimental results of our first paper have been confirmed by other authors since then, and, as we shall presently point out, are further confirmed by the table given by Copeman and Hake.

The theory held regarding the diminution in hydrochloric acid in *carcinoma ventriculi* previously to our first paper, was that the cause was local in the stomach itself. In the light of our experiments showing similar results with the growth situated in organs remote from the stomach this view became untenable, and we referred the reduced acidity to a general condition in the blood.

Further experimentation, the results of which were published in a second paper, showed us that *the degree of acidity of the gastric contents is most sensitive in many diseased conditions*, and under an hospital régime; and we gave results showing that in twenty hospital cases, where there was no cancer, the average amount of hydrochloric acid was much reduced below the average normal amount in healthy persons.[1]

1. Similar results were obtained independently and practically simultaneously by Morton Palmer (*loc. cit.*).

This point regarding the diminution in hydrochloric acid in non-malignant cases we especially emphasized, and placed as the chief result in the forefront of our conclusions. Reflections on the importance of the hydrochloric acid as a peptonizing agent, as a disinfectant of the alimentary tract and of the food, and as an excitant to the hormone (secretin) for the pancreatic secretion, clearly demonstrate the importance of this depreciation of the hydrochloric acid value as a general factor in disease, and in predisposing to infection; but upon this we need not further dilate here than to remark that we regarded the observation as an important one entirely apart from the question of malignant disease.

So far were we from regarding hydrochloric acid as being absent in *all* cases of malignant disease, or its absence as a diagnostic sign, that we safeguarded ourselves by specially pointing this out in the following words:—

'It is clear from these figures that the presence or absence of free hydrochloric acid cannot be regarded as a diagnostic sign of any great value. All that can be said is that in the majority of malignant cases it is absent, and in the majority of non-malignant cases it is present. The same statement holds for cancer of the stomach wall itself.'

That the amount of hydrochloric acid is more reduced in malignant than in non-malignant cases is, however, clearly shown by the following summary extracted from our second paper:—

		Malignant		Non-malignant		Ratio
'Total acidity	=	816	:	1,453	=	0·56
Di-methyl indicator	=	352	:	925	=	0·38
Günzburg's reagent	=	163	:	502	=	0·32
Methyl-acetate method	=	286	:	630	=	0·45
Mörner-Sjoqvist method	=	637	:	774	=	0·82'[1]

It is entirely an open question whether this greater drop in acidity in the malignant cases is due to the greater severity of the illness and the accompanying cachexia, or is a specific effect in cancer; but in any case it is obvious that it must be referred to a general cause in the system, and not to local conditions in the stomach.

1. The reason for the nearer approach to equality in the Mörner-Sjoqvist method is discussed in our paper and shown to be the presence of organic base in combination with inorganic acid.

Having thus defined the chief experimental results given in our previous papers, I may now turn to the criticisms of Copeman and Hake, and in the first place will reply to certain criticisms of our technique and methods.

It is implied, because we do not explicitly state that no water was added when we were drawing off the test meal at the end of the period, that we probably added water—especially because Copeman and Hake found difficulty in the majority of their cases in getting the stomach contents off without water.

Since the contents were being drawn off in order to make *quantitative* estimations of the hydrochloric acid, it would scarcely seem necessary to a chemist to state that no water was added, but as it appears to be necessary I may now say that the contents were taken without the addition of water. The enormous difficulty experienced by Copeman and Hake was not encountered in doing this, and we believe that we are correct in stating that the withdrawal of stomach contents without adding water has been, and often is, accomplished by others than ourselves. As evidence of the low results for amount of acid in our cases being caused by too much added fluid, Copeman and Hake quoted one case of ours (No. IX) in which, under special circumstances, we gave three pints of test meal instead of one, and withdrew it after one and a half hours, and they state that ‘ it is hardly surprising that under these circumstances the free hydrochloric acid obtained was only 0·001 per cent.’ In reply to this I would state that if Messrs. Copeman and Hake will take the trouble to administer a pint of gruel and two pints of water in normal individuals, and withdraw after the same interval of one and a half hours, I should be very much surprised indeed if they did not get more than fifty times as great an amount of hydrochloric acid.

They appear to forget that the physiological experiments show that the acid secretion is regulated by the concentration of acid in the stomach. Fluid added at the time of withdrawal is, of course, a different matter.

With the exception of this one case, in which the amount of fluid

given is definitely stated, the amount given was one pint, and it was withdrawn at the end without adding water.

Messrs. Copeman and Hake next proceed to criticize very adversely the variations in time before we withdrew our test meals for analysis, as follows :—

'It is of importance to note, also, that the interval between the administration of the test meals and its withdrawal, as recorded by these observers, varied between the wide ranges of one to two hours, and in a single case was as much as three hours. This absence of uniformity as regards time of withdrawal clearly detracts from the diagnostic value of the results, and it is sufficiently obvious that where a number of experiments are being made under varying conditions of time, whether by one or more observers, no proper comparison will be possible between the results or the deductions drawn from them.'

I regard this statement *as applied to our experiments* as an unfair criticism, which is rendered all the more misleading by the abstract truth of its concluding sentence.

In the first place we did not claim 'diagnostic value' for our results, and explicitly say so in our discussion (p. 155) ; but this is a minor matter. The obvious meaning that any reader would draw from the above quotation is that we collected our test meals at very varying intervals indiscriminately from one hour to two hours, and in one case up to three hours, so rendering them quite valueless. Let us consider first the single extreme three-hour interval. No mention is made by our critics of the fact that this observation is bracketed in our table with another observation and series of analyses done *on this same patient after an exact interval of one hour.* This is the only case in the entire series where two observations are taken on the same patient, a fact which could scarcely escape the attention even of a casual reader of our table. We may now further draw our critics' attention to the important fact, which also appears to have escaped their notice, that the two sets of analyses in this case practically coincide after one hour and after three hours respectively, the figures for total acidity being 0·2665 per cent. after three hours, and 0·2847

per cent. after one hour; Günzburg reaction negative in both analyses; free hydrogen ion concentration by methyl-acetate method = 0·00067 per cent. HCl for three hours, and zero HCl for one hour.

Turning to the more serious statement that our times of collection ranged from one to two hours, a glance at the times recorded in our table[1] shows that of thirty-three cases the test meal was siphoned off in *exactly* one hour in twenty cases; in five cases the period was one and a quarter hours; in six cases one and a half hours; while *in two cases only was the interval two hours.* Considering that these are clinical experiments, where the meal is administered and the withdrawal carried out in the ward, I am inclined to think that they were carried out as regards time in a fairly uniform and scientific manner; nor do the results show any variations such as would warrant the criticism quoted above. Within these time limits there is no obvious variation, related to time, visible in the results.

With this I may conclude my reply to Messrs. Copeman and Hake's criticism of our previous papers, and turn now to a criticism of their paper and results.

The first section of the paper deals with the acidity of the stomachs and stomach contents of mice which had carried implanted tumours, and demands only one or two passing references.

It is not surprising to me to find the result stated in this section that the amount of hydrochloric acid in the gastric contents in mice bearing implanted tumours is not diminished, as this adds but one more proof, if such were needed, that these interesting growths, even if carcinomata, are carcinomata situated in healthy animals where the conditions are such that the animal reacts against the effects of the growth, and so produces an immunity which is shown by the absence of general effects in the organism.

Anyone who has stood and watched mice, as I have done, with tumours of this class nearly as large as themselves, must have been struck with the obvious fact that there are none of those clinical characteristics of cancer as observed in man to be seen in the behaviour of the animals.

1. *Bio-Chem. Journ.*, Vol. I, pp. 276 and 277.

The mice are to all appearances perfectly well; they eat with all the vigour of the healthy mouse, they run about and clean themselves like the normal mice; there is no cachexia or wasting unless the tumour breaks down owing to its size or to injury.

Attention has been drawn to the absence of cachexia in mice with implanted tumours by Dr. Bashford, the Director of the Imperial Cancer Research, who, as quoted by Copeman and Hake in the very paper we are criticizing, states that on the basis of observations made on almost 3,000 mice with propagated cancerous tumours during two years 'the conclusion has been arrived at that the presence of a tumour even of greater weight than the mouse itself does not necessarily involve a disturbance of the normal nutrition which could be regarded as comparable to the cachexia frequently associated with malignant new growths in the human subject.'[1]

In view of these important differences between experimentally implanted tumours in mice and malignant growths in man, I desire to enter a protest against their being described as 'cancerous' tumours and 'cancerous' growths, and the mice being described as 'cancer' mice, in the same sense as these terms are used in regard to the human subject, until the causes of the differences have been cleared up.

It is only in a small percentage of cases that these experimental tumours give rise to metastatic growths, viz., 4 to 5 per cent., whereas in cancer it is only a small percentage which escape metastatic growths.

We are told by Copeman and Hake that 'recent extensive statistics, collected by the Imperial Cancer Research Fund from various London hospitals, have shown that cachexia is not a constant accompaniment of cancer in man.'

Perhaps cachexia is not a *constant* accompaniment of cancer in man any more than the diminution or absence of hydrochloric acid in the gastric contents is a *constant* accompaniment—few or no clinical symptoms or signs are absolutely constant accompaniments of any disease. But a research into the cause of death in cancer unaccompanied by cachexia would be of high scientific interest,

1. *Scientific Reports on the Investigations of the Imperial Cancer Research Fund*, No. 2, p. 40, 1905.

and any such highly interesting cases should be watched to the end. Loss of appetite, wasting and anaemia, with their related conditions of mind and body, are prominent symptoms in many early cases of internal cancer long before a tumour can be located, and certainly before there is ulcerative breaking down of tissue, and ordinary septic toxaemia. In such cases it is possible that the normal tissue cells are being poisoned by toxic substances derived from the cancer cells, and chemical research is much needed to clear up this and similar problems.

In our second paper we showed that there was evidence, from certain of the methods which we employed, that the gastric contents in cancer cases contained organic bases.

The results obtained by Copeman and Hake with the Volhard-Lüttke method prove, as we shall presently point out, that chlorides of such bases existed in the gastric contents from their cases.

Further, over 10 per cent. of the nitrogen of the urine in cancer patients is present as extractive nitrogen, an amount far in excess of what is ever found in health.

Experimental work on the subject of these organic bases is at present being carried out in the Bio-Chemical Laboratory, Liverpool.

It may well be that the absence or diminution of free hydrochloric acid in cancer is closely associated with the cachectic condition, although it often precedes its obvious onset, and if this is the case, it is easy to understand why there is no decrease in tumour mice, because they are never cachectic, and seem to suffer no change in their general health from the presence of the tumour.

We may now turn to the results obtained by Copeman and Hake in the cases in which quantitative determinations of acidity were made in human cases.

Only thirteen cases are described in their table in which the test meal was obtained without dilution with water, and as details are not given regarding the other cases of their thirty-four, we may confine our attention to these tabulated cases.

Three cases of the thirteen are shown by the results to contain

about a normal amount of hydrochloric acid as compared with the single normal control, viz., those numbered V, VII, and XXXI; in the other ten, in my opinion, the figures show that the free hydrochloric acid is very considerably decreased, and in several it is practically absent.

Taking the methods in order, the total acidity to phenol-phthalëin is not markedly reduced, the average of the thirteen cases showing 0·1636 per cent., while the single normal case gave 0·1861; but it is peculiar that while the total acidity to an indicator which will show the faintest trace even of a feeble organic acid is 0·1861 per cent., the 'physiologically active hydrochloric acid' alone amounts to 0·2336 per cent.; that is to very considerably more than the total acid. It may be suggested that there is something wrong with a method which shows 'physiologically active' hydrochloric acid that cannot affect phenol-phthalëin. This result is also given in no less than nine of the thirteen cancer cases. Surely it would be more appropriate, if chlorine which is so firmly attached to base is to be termed hydrochloric acid at all, that it should be designated as physiologically *inactive* hydrochloric acid—it is just about as physiologically active as sodium chloride.

When we pass to the column of the table which shows the reaction to the Günsburg reagent, it becomes obvious that the amount of free hydrochloric acid in the majority of the cases is quite inappreciable. Thus in four of the cases the result is absolutely negative, being marked in the table by 'nil'; in three other cases it is noted as 'faint'; and since the authors state in the text that 'where a very marked reaction was obtained we also made the test quantitatively,' it may be taken that in these seven of the thirteen cases the amount of free hydrochloric acid shown by this most reliable test was too infinitesimal to estimate. That is to say, in more than half the cases there was practically no free hydrochloric acid present. In two other cases the result is given as 'very marked' and 'marked,' but no estimation is made—these two belong to the three cases which we have admitted above as exceptions out of the total thirteen cases. In only four of the thirteen cases is a quantitative determination of

the free hydrochloric acid made by the Günsburg reagent, and from the above quotation from the text it may be taken that these were 'very marked' reactions; the average of the four works out to 0·0496 per cent., the control being 0·1314 per cent. If we add the seven cases where the amount was too low to estimate, the average for these eleven cases has the value 0·0180 per cent., and I do not desire any better confirmation of our figures than this.

If this does not mean a marked reduction in free hydrochloric acid in these cancer cases, I entirely fail to understand what it does mean.

The concentration of free hydrogen ions as shown by velocity of inversion of methyl-acetate is worked out in all of the thirteen cases. This method gives quantitatively the effective acidity for physiological work of the gastric contents, and that which would be most closely connected with its digestive and bactericidal properties, and hence might much more appropriately be taken as showing 'physiologically active hydrochloric acid' in the true sense of the words. The results obtained by Copeman and Hake by the use of it, again entirely support our conclusion as to the depression in acidity in cancer. Thus the average for the whole thirteen cases is 0·0407 per cent. as against 0·1195 per cent. in the control case, or about one-third of the value, and if we take out the three cases which are admitted exceptions, we obtain for the remaining ten cases the average value of 0·0153 per cent., which I regard again as a marked reduction from the normal percentage.

The two remaining methods used by Copeman and Hake are the Mörner-Sjoqvist and the Volhard-Lüttke methods; these two methods are almost identical in principle, and are subject to the same errors.

The Mörner-Sjoqvist method consists essentially in incinerating in presence of excess of barium carbonate, when any free hydrochloric acid *plus* any hydrochloric acid, however firmly combined with organic bases, is converted into barium chloride, and the amount of this estimated in the solution obtained from the incinerated mass. The method introduced by Lüttke consists in making two estima-

tions of chlorides in two incinerations, in one of which acid free or organically combined is driven off on the incineration, and thus does not appear in the analysis, and in the second incineration the addition of alkali to neutralization to phenol-phthalein prevents the loss of any chloride save such as disappears owing to volatilization in the removal of the charred organic matter. The difference in the two figures hence is supposed here as in the Mörner-Sjoqvist method to give free hydrochloric acid *plus* hydrochloric acid combined with organic bases.

The Mörner-Sjoqvist method is by far the better of these two, since there is much less danger of loss due to volatilization of chlorine on incinerating such a small amount of sodium chloride in presence of such an excess of organic matter. It was for this reason, and on account of the great similarity of the two that we used the Mörner-Sjoqvist method in our work.

In our paper we drew attention to the fact that the Mörner-Sjoqvist method, as well as another method of an allied nature which we used to attempt to determine the amount of organic acid, viz., incinerating with excess of alkali, gave figures which indicated a considerable amount of organic base in the gastric contents, and we drew special attention to the importance of the presence of this amount of organic bases.

Thus we found that incinerating with alkali, instead of giving rise to positive figures owing to destruction of organic acids, gave often negative figures indicating that alkali was used up, and having shown that ammonia was practically absent by Schlössing's method, as well as other volatile nitrogenous bases, we were driven to the conclusion that fixed organic bases were present in more than sufficient amount to preponderate and mask any organic acids which might be simultaneously present.

This very important result has not, in my opinion, yet received the attention which it deserves, and is worthy of further investigation to determine the nature and physiological properties of these basic bodies. That they are not proteins or first products of the hydrolytic cleavage of proteins by digestion in the stomach, is shown by the fact

that they neutralize the hydrochloric acid to methyl orange and di-methyl, and even to phenol-phthalein, as also by the fact that only a very weak biuret reaction is given by such samples of stomach contents upon a test meal.

To speak of such firmly combined chlorides as 'physiologically active hydrochloric acid' is, for the reasons above mentioned, peculiarly absurd. Nor does it matter that the acid so combined may previously have been free, as suggested by Copeman and Hake, for after combination it is no longer either chemically or physiologically free or active. Further, as has been pointed out already, under normal conditions of gastric secretion, acid is secreted until the free acid present (or rather the hydrogen ion concentration) approximates to a normal value, so that any alkali would be neutralized, and then free acid would go on being secreted until the normal value of acidity was once more attained.

Hence the figures obtained by Copeman and Hake by the Lüttke method chiefly show firmly combined organic chlorides, and not physiologically active hydrochloric acid, and serve to confirm the results previously obtained by us using the Mörner-Sjoqvist method, and that of incineration after neutralization or with excess of alkali.

Since our principal results have now received confirmation by Morton Palmer, by Willcox, and, in my opinion, by the figures of Copeman and Hake themselves, I still adhere to them, and in conclusion would definitely state them as follows :—

1. The free hydrochloric acid of the gastric contents after a test meal is extremely sensitive in most diseased or debilitated conditions, and the average of a series of cases taken at random from a hospital ward is lower than a similar series from healthy individuals.

2. The depression is particularly well marked in cases of cancer, and this is found to hold wherever the cancer growth happens to be situated. This shows that the very low concentration of hydrochloric acid in cancer is not due, as formerly supposed, to local conditions in the stomach, but must be referred to some general change in the blood affecting the hydrochloric acid secretion. In this connection it may be added that there is a small but distinct increase in the

alkalinity of the salts of the serum in cases of cancer (Moore and Wilson).[1]

3. It is at present indeterminate whether the greater drop in cancer cases is due to the greater degree of cachexia and general ill-health in such cases, or whether it is due to something specific in cancer.

4. In addition to the depression in free hydrochloric acid, there is also a considerable amount of bases in combination as chlorides, and these bases do not consist of ammonia or other volatile nitrogenous bases, or of proteins or primary products of protein cleavage, but of organic bases.

5. The depression in free hydrochloric acid occurs in the majority of cases, but there are exceptions to the rule, and for this reason, and also because hydrochloric acid may be absent or reduced in other conditions than cancer, the absence or diminution can not be taken as a diagnostic sign of any very great value.

1. *Bio-Chem. Journ.*, Vol. I, p. 297, 1906.

THE HYDROLYTIC ENZYMES OF INVERTEBRATES

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The object of this research was to test for hydrolytic enzymes in the invertebrate kingdom, and to investigate the conditions of optimum activity of any proteoclastic enzymes found.

The number of papers dealing with the digestive processes of invertebrates is so great, and as they mostly deal with qualitative measurements, it is beyond the scope of this paper to review the literature[1] except where there are points to which it is especially necessary to refer.

Methods

The enzymes were prepared by treating the minced tissue, of which it was desired to test the enzymic activity, with an equal weight of glycerine. This was allowed to stand for several weeks, and then the solid portions were removed by straining through cotton. In later experiments an attempt to purify these extracts was made by precipitating the glycerine extract with alcohol, and after filtering off the precipitate it was extracted with water containing a little toluol.

In all the experiments the action of the enzyme was controlled by an identical mixture, using some of the enzyme extract in which the activity had been destroyed by boiling. The ratio of the enzyme extract to the volume of the digestion mixture was 1 : 10.

Most of the extracts were made during the month of April in the years 1907 and 1908.

1. For the literature up to 1903 see O. v. Furth, *Vergleichende chemische Physiologie der niederen Tiere* (Jena, Gustav Fischer).

Carbohydrate Hydrolysis

Table I shows the results of the action of fresh glycerine extracts on carbohydrates. Two per cent. solutions of the different carbohydrates were taken, and to each 9 c.c. 1 c.c. of enzyme extract was added. This gave an initial concentration of 1·8 per cent. of the carbohydrate. At the end of the experiment a known volume was heated with excess of Fehling's solution, and the cuprous oxide was filtered on to a Gooch crucible, washed, oxydised, and weighed as cupric oxide. The figures give the percentage of glucose formed. In the case of maltose the amount of glucose is determined from the difference in reducing power between the active enzyme and the control. In the other experiments on starch, glycogen, and saccharose, the controls were, of course, negative. The digestion was allowed to proceed for a period of forty-eight hours at 25° C.

Table I

Source of Enzyme	Starch	Glycogen	Saccharose	Maltose
Digestive gland of *Cancer pagurus* (edible crab)	0·51	0·43	0·30	0·48
' Liver ' of *Patella vulgata* (limpet)	0·72	0·59	0·98	0·42
Intestine (oesophagus and inferior spiral) of *Echinus esculentus* (sea urchin)	0·98	0·54	1·25	0·63
' Disc ' of *Ophiocoma nigra* (brittle star)	0·31	0·04	0·83	—

The experiments recorded in Table II were performed with the enzymes prepared by precipitation of the glycerine extracts with alcohol. Owing to length of time required to show the presence of enzymes capable of hydrolysing maltose and lactose it was found impossible to conduct a long series of experiments by the usual methods, but by using Barfoed's reagent the change from di-saccharide to mono-saccharide can be easily detected.[1] The hydrolysis of poly-saccharides was proved by the disappearance of the colour given with iodine, and by reduction of copper in alkaline solution. The decomposition of saccharose into mono-saccharides was found by the appearance of reduction on heating with an alkaline copper solution.

The table shows the lengths of time before appreciable hydrolysis occurred ; the temperature at which the experiments were conducted was 27° C.

1. Roaf, *Bio-Chem. Journ.*, Vol. III, p. 182, 1908.

Table II

Source of Enzyme	Starch	Glycogen	Saccharose	Lactose	Maltose
	Time required to show appreciable hydrolysis				
Digestive gland of *Cancer pagurus* (edible crab)	22 hours	22 hours	6 days	8 days	22 hours
'Liver' of *Patella vulgata* (limpet)	16 „	16 „	4 „	3 „	16 „
'Liver' of *Pecten opercularis* (scallop)	18 „	18 „	42 hours	18 hours	18 „
Intestine (oesophagus and inferior spiral) of *Echinus esculentus* (sea urchin)	20 „	20 „	No hydrolysis	6 days	68 „
Mesenteric filaments of *Tealia crassicornis* (sea anemone)	66 „	No hydrolysis	No hydrolysis	No hydrolysis	39 „
Mesenteric filaments of *Actinia mesembryanthemum* (sea anemone)	No hydrolysis	No hydrolysis	No hydrolysis	No hydrolysis	96 „
'Disc' of *Ophiocoma nigra* (brittle star)	—	No hydrolysis	24 hours	6 days	—

In addition it was found that the extract of the whole colony of *Cellaria fistulosa* (polyzoon) hydrolyses starch, and the extract of the 'pyloric caeca' of *Asterias rubens* (starfish) also acts on starch although the 'stomach' extract of the same organism is without action.

Table III

Source of Enzyme	Hydrochloric Acid $\frac{n}{20}$	Hydrochloric Acid $\frac{n}{25}$	Hydrochloric Acid $\frac{n}{40}$	Hydrochloric Acid $\frac{n}{80}$	Neutral	Sodium Carbonate $\frac{n}{80}$	Sodium Carbonate $\frac{n}{40}$	Sodium Carbonate $\frac{n}{25}$	Sodium Carbonate $\frac{n}{20}$	Effect of Temperature: Reaction	11°C.	25°C.	35°C.
Digestive gland of *Cancer pagurus* (edible crab)	—	—	0·9	—	3·1	3·6	3·4	4·0	**4·2**	$\frac{n}{20}$ Na_2CO_3	1·5	3·8	4·3
'Liver' of *Patella vulgata* (limpet)	2·5	3·8	**5·4**	4·3	0·5	—	1·8	—	—	$\frac{n}{40}$ HCl	3·7	5·5	6·3
Intestine (oesophagus and inferior spiral) of *Echinus esculentus* (sea urchin)	1·0	1·1	**2·4**	0·5	0·1	—	—	—	—	$\frac{n}{40}$ HCl	1·6	2·4	2·1
'Disc' of *Ophiocoma nigra* (brittle star)	—	0·5	**1·5**	1·4	0·4	—	—	—	1·4	—	—	—	—
Mesenteric filaments of *Tealia crassicornis* (sea anemone)	—	1·4	0·6	0·1	4·8	—	4·8	—	**5·5**	—	—	—	—

Protein hydrolysis was found to be present in both acid and alkali using the extract of the 'pyloric caeca' of *Asterias rubens* (starfish), but the stomach extract was without effect in either reaction. Extracts of the whole colonies of *Alcyonium digitatum* (dead man's toes), *Cliona celata* (boring sponge) and *Cellaria fistulosa* (polyzoon) showed protein digestion in acid reaction but not in alkaline media, and the extract of the mesenteric filaments of *Actinia mesembryanthemum* (sea anemone) in $\frac{n}{40}$ HCl showed increasing action with temperatures up to 35° C.

Protein Hydrolysis

Protein hydrolysis was investigated, using fibrin as a substrate. The first series of experiments was made by keeping the digestive mixtures for forty-eight hours at 25° C., and at the end of that time 10 c.c. of 10 per cent. tri-chloracetic acid was added to each 20 c.c. This was then filtered, and the nitrogen determined in 10 c.c. of the filtrate by the method of Kjeldahl. The control figures were subtracted from those given by the active enzymes, and the differences in cubic centimetres of decinormal acid are given in Table III. The first portion of the table shows the effect of digestion at 25° C. for forty-eight hours, with varying reaction, and the second portion shows the effect of varying the temperature when the reaction is kept constant.

In later experiments the rate of digestion was estimated by the coloration produced by the solution of congo red fibrin.[1] In order to test if this method gave the maxima of digestion in the same strengths of acid and alkali as the usually accepted methods, an experiment was carried out with ordinary pepsin and trypsin at 40° C. The results showed that the maximum occurs with pepsin in, at least, $\frac{n}{20}$HCl (0·18 per cent.), and with trypsin in $\frac{n}{5}$ Na_2CO_3 (1·06 per cent.).

The congo red fibrin method was then applied to a series of extracts (Table IV). It was thought that perhaps the protein present would interfere with the digestion of fibrin, so in all except two experiments the enzyme was prepared by precipitation of the glycerine extract with alcohol, and extraction of the alcohol precipitate with toluol water. It was found that the enzyme in the case of certain extracts was apparently altered so that digestion now occurred better in sodium carbonate than in hydrochloric acid (compare Tables III and IV).

1. Roaf, *Bio-Chem. Journ.*, Vol. III, p. 188, 1908.

Table IV

Source of Enzyme	Hydrochloric Acid $\frac{n}{20}$	Hydrochloric Acid $\frac{n}{25}$	Hydrochloric Acid $\frac{n}{50}$	Hydrochloric Acid $\frac{n}{100}$	Neutral	Sodium Carbonate $\frac{n}{100}$	Sodium Carbonate $\frac{n}{40}$	Sodium Carbonate $\frac{n}{20}$	Sodium Carbonate $\frac{n}{10}$	Sodium Carbonate $\frac{n}{5}$	Duration and Temperature
Digestive gland of *Cancer pagurus* (edible crab)	—	—	—	—	—	20	35	60[1]	60	20[2]	4 hours at 40° C.
	—	—	—	—	15	40	40	10	10	10[2]	4 hours at 37° C.
' Liver ' of *Patella vulgata* (limpet)	10	30	—	—	—	—	—	—	—	—[2]	6 hours at 40° C.
	—	—	—	—	—	—	10	30	30	25	65 hours at 27° C.
	10	15	10	—	—	—	40	?	20	15	48 hours at 40° C.
' Liver ' of *Pecten opercularis* (scallop)	—	—	—	—	—	10	25	30	50	40	42 hours at 27° C.
Intestine (oesophagus and inferior spiral) of *Echinus esculentus* (sea urchin)...	—	—	—	—	—	40	10	12	—	—	48 hours at 27° C.
Mesenteric filaments of *Tealia crassicornis* (sea anemone)	—	—	—	—	—	—	15	10	—	—	66 hours at 27° C.
Mesenteric filaments of *Actinia mesembryanthemum* (sea anemone)... ...	—	—	—	—	—	10	30	15	10	—	72 hours at 27° C.

The figures show the amount to which the digestion mixture must be diluted in order to give solutions of equal depth of colour ; where the digestion was absent or so slight as to give no appreciable coloration the space is left blank.

1. With $\frac{n}{20}$ Na_2CO_3 the digestion at 15° = 10 c.c. to match which a similar digestion at 40° had to be diluted to 80 c.c.

2. These experiments were performed with the fresh glycerine extract.

Fat Hydrolysis and Coagulating Enzymes

The action of the enzymes on boiled milk containing litmus was also investigated. Some experiments were performed with the fresh glycerine extract, and some with the extract further prepared as already described. In nearly all cases coagulation occurred,[1] but in order to notice this result it was often necessary to observe carefully as the coagulum became rapidly digested. Coagulation failed in only one case, and there it was quite evident that the caseinogen had become hydrolysed into simple bodies. The purified enzyme in these cases caused a precipitate, and not a solid coagulum. This was apparently due to the conditions of the experiment, as the enzyme from the same source, before preparation, caused a coagulum. In all cases except one the litmus was turned red, showing the production of acid. In that case the enzyme was one which had been precipitated with alcohol, and the fresh enzyme from the same source caused the development of acidity. The coagulation was not merely due to the development of acid.

The fresh glycerine extracts were tested on oxalated blood plasma. In several cases a distinct coagulation occurred, and in others a curdy precipitate not unlike fibrin appeared. As the observations were complicated by digestion of fibrin, it was difficult to determine whether the coagulation was absent or only masked by digestion of the resulting fibrin. At present no explanation of this result can be given, but it is recorded here in the hope that it may be of some use, and the effect is being investigated further.

The results of the action on litmus milk and blood plasma are given in Table V :—

1. A rennin-like enzyme has been found in *Suberitas domuncula* (Colte, *Soc. biol.*, Vol. LIII, p. 95, 1901); in *Cancer pagurus* and *Maia squinado* (Sellier, *Soc. biol.*, Vol. LXI, p. 449, 1906, and Vol. LXIII, p. 703, 1907); in *Aphrodite aculeata* (Sellier, *Soc. biol.*, Vol. LXII, p. 693, 1907); and in *Cambarus* (Bradley, *Proc. Amer. Soc. Biol. Chem.*, p. 36, *Journ. Biol. Chem.*, Vol. IV, 1908).

Table V

Source of Enzyme	Blue litmus milk		Oxalated blood plasma
Digestive gland of *Cancer pagurus* (edible crab)	Coagulated	Turned red[1]	Not clotted
'Liver' of *Patella vulgata* (limpet)	Coagulated	Turned red[1]	Jelly-like coagulum
	Precipitated	Turned red[2]	
'Liver' of *Pecten opercularis* (scallop)	Precipitated	Turned red[2]	Curdy precipitate-like fibrin
Crystalline style of *Pecten opercularis* (scallop)			Jelly-like clot
'Liver' of *Pupura lapillus* (dog whelk)			Curdy precipitate-like fibrin
'Liver' of *Trochus zizyphinus* (top shell)			Slight precipitate
'Liver' of *Buccinum undatum* (whelk)			Slight precipitate
Intestine of *Echinus esculentus* (sea urchin)	Coagulated	Turned red[1]	
	Coagulated	Turned red[2]	Jelly-like coagulum
'Disc' of *Ophiocoma nigra* (brittle star)	Coagulated	Turned red[1]	Slightly jelly-like
Pyloric caeca of *Asterias rubens* (starfish)	Digested	Turned red[1]	Curdy precipitate-like fibrin
Mesenteric filaments of *Tealia crassicornis* (sea anemone)	Coagulated and then rapidly digested	Turned red[1]	Coagulated
	Precipitated and then digested	Not turned red[2]	
Mesenteric filaments of *Actinia mesembryanthemum* (sea anemone)	Coagulated	Turned red[1]	Curdy precipitate-like fibrin
Alcyonium digitatum (dead man's toes)			Coagulated
Benger's liquor pancreaticus			Jelly-like coagulum

1. Fresh glycerine extract.
2. Extract prepared from alcohol precipitate of glycerine extract.

Discussion of Results

In considering the results of experiments on enzymes a positive result is definite, but a negative result only shows the absence of the particular enzyme sought under the conditions of experiment. A different method of extracting the tissue, or altered experimental procedure, may show an enzyme where none had previously been found. However, in the experiments here recorded it is seen that glycerine can extract all the enzymes from certain organisms, so that absence in other cases may be construed as showing absence of the missing enzyme.

In the digestion of carbohydrates it is seen that the fresh glycerine extracts (Table I) show, where any enzyme is present, all the various actions—except in the case of Ophiocoma, where the glycogen-splitting enzyme is practically absent. It is also seen that although the pyloric caeca of Asterias digests starch, the stomach is without action. With the enzymes prepared by alcohol precipitation (Table II), it is seen that Ophiocoma again fails to hydrolyse glycogen. The only difference from the fresh enzymes is that Echinus fails to act on saccharose. Tealia acts only on starch and maltose, whilst the allied species *Actinia mesembryanthemum* only hydrolyses maltose. It is interesting to note that Giaja[1] has found a large number of actions on glucosides and carbohydrates from various marine crustacea, and thus one is led to suppose that the number of enzymes must be infinite, or else that one enzyme can perform a variety of actions limited only to a certain extent so that one action is less marked or negative according to the stereochemical relationships of the substrate.

The question of proteoclastic activity is much more complicated, as not only the presence of an enzyme but also the optimum conditions of action must be determined. In some cases this has been fairly well determined, but in others, partly owing to difficulty in obtaining a sufficient supply of active enzyme, only a few orientating points have been determined. Thus in Alcyonium, Cliona, and Cellaria an enzyme active in acid has been found; in Asterias activity in both

1. *Soc. Biol.*, Vol. LXIII, p. 508, 1907.

acid and alkali has been shown with the pyloric caeca, but the stomach (as with starch) has been found inactive.[1] These measurements were qualitative, but not quantitative, whilst for the other enzymes quantitative estimations have been made.

Effect of Temperature.—Increase of temperature up to 35° C. causes a more rapid hydrolytic action. This has been previously observed by several workers, and is simply the usual increase of velocity with increasing temperature. The maximum is reached when the temperature is high enough to cause sufficient destruction of enzyme to overbalance the increasing rate of reaction.

Effect of Reaction.—Krukenberg in various instances states that there are two proteoclastic enzymes present, one acting in acid, and the other in alkali. In several extracts this has been confirmed (Patella, Ophiocoma, Tealia), but it is usual to find that one action is much stronger than the other. Precipitation with alcohol, by removing protein, lowers the strength of acid or alkali requisite for optimum activity.[2]

With Patella, alcohol precipitation changes the more effective reaction from acid to alkali, and with Echinus the action in acid disappears; but, unfortunately, the effect in alkali had not been tested before precipitation.[3]

The most efficient strength is much nearer the neutral point than with mammalian pepsin or trypsin. The maxima of acidity and alkalinity would depend on the temperature, as rise of temperature would lead to increased ionisation, and hence greater effective acidity or alkalinity, and possibly greater destruction of enzymes.

The presence of a rennin-like enzyme has been observed in various invertebrata by several workers, and lipase has also been found to occur widely distributed in plants and animals. The

1. Previous workers also find the pyloric caeca active and stomach inactive, v. Furth, *loc. cit.*, p. 168.

2. To cause the congo red fibrin to turn blue requires from $\frac{n}{50} - \frac{n}{20}$ HCl, whilst with congo red in distilled water $\frac{n}{1000}$ HCl is sufficient, thus showing the inhibiting action of fibrin (and other proteins) to change of reaction.

3. Whether this is due to two enzymes which are differently affected by the method of preparation, as shown by Hedin for proteases of spleen (*Journ. Physiol.*, Vol. XXX, p. 155, 1904), must be determined by further investigation.

coagulation of oxalate plasma requires further investigation, but the action is not likely to be due to a kinase, as, in the absence of calcium salts, the kinase would not be able to produce thrombin from thrombogen. Hence it appears as if there were already present in the extracts some substance capable of acting to a certain extent like thrombin.

Development of Selective Action

The action of enzymes on stereochemically allied substances suggests that one enzyme is responsible for more than one action. The range of substances acted upon by an enzyme would depend on the degree of specialisation to which that enzyme had attained. Thus there are several explanations as to the relationship between pepsin and rennin. There may be two separate enzymes, or one enzyme with two different active groups; or the rennin action may be only the first stage of the hydrolytic action of pepsin. In certain cases the last explanation seems most applicable, because the presence of a rennin-like enzyme in plants and non-mammalian animals cannot be normally used for coagulating milk, but must be associated with some other function. That a purely hydrolytic action can clot milk can be shown by the action of alkali. Quite dilute sodium hydrate (sufficient to make the added alkali up to 0·16 per cent.) causes a coagulation of caseinogen, and with slightly stronger solutions the caseinogen is further hydrolysed, and re-dissolves. The duration of these processes depends on the strength of alkali and the temperature. All stages, from simple coagulation to a coagulation followed by rapid solution, have been observed.

The principle of evolution, when applied in its broadest sense, should show a gradual transition of chemical composition accompanying the changes in structure. As applied to enzymes one should expect that the original forms of enzymes would be less specialised, acting somewhat like an acid or an alkali, and that as the animal (or plant) became specialised its enzymes would become differentiated, so that some of them could only act on certain definite substrates. This would be accompanied by development of some special molecular

group, so that finally the solubility and other properties of the specialised enzyme might differ greatly from the original form in which the enzyme existed.

In such a scheme we would expect to find, in connection with proteoclastic enzymes, a series of enzymes ranging from those capable of acting equally in weakly acid or alkaline media, through those which act better in one reaction than another, to the more specialised ones occurring in the mammalian body, such as pepsin, trypsin, rennin, erepsin, amylopsin, thrombin, etc. On this view the question of the identity of pepsin and rennin might depend for its answer on what particular organism was selected for investigation. In one animal the two might have become differentiated, whilst in another their two actions might be bound up together. Likewise, the enzymes found in any one species would depend on the nature of the food material to which that species was accustomed, lesser action on substances allied to its food, and the less specific the enzymes were the wider, though probably less intense, their action. Some such reason must explain the presence of rennin, lactose, and possibly thrombin in organisms which normally have no need for those actions.[1] The presence of proteoclysis in both acid and alkali may not be due to two enzymes, but to one, and by various treatment one or other of these actions may be diminished, thus giving rise to some of the changes in action noted in these experiments after precipitation by alcohol.

1. Krukenberg (*Vergleich-physiologische Vorträge*, p. 72, 1886) stated that digestion in all animals is potentially one and the same.

VARIATIONS IN THE OSMOTIC CONCENTRATION OF THE BLOOD AND COELOMIC FLUIDS OF AQUATIC ANIMALS, CAUSED BY CHANGES IN THE EXTERNAL MEDIUM

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In a previous paper in this journal, the results of a series of experiments were given, made to determine to what extent the constitution of the blood of fishes was dependent upon the concentration of the water, sea or fresh, bathing their bodies.

It was shown then, that although the teleostei maintained an almost constant osmotic pressure of the blood, which was only about one-third that of sea water from the open sea, yet at the same time they were slightly dependent upon the outer medium, and the blood was not absolutely unaffected by changes in the latter as affirmed by many writers. The concentration of elasmobranch blood was on the other hand practically identical with the sea water.

The following is a continuation of this work; and though the conditions prevailing in invertebrates has also been considered, they will only be touched upon here since a very good summary of researches on osmotic pressure has been published this year by Bottazzi.[1]

The study of the physico-chemical conditions of the salts of the blood and internal media of the body, can be followed along three closely related paths dealing with:—(1) The relation of the constitution of the blood of aquatic animals to the medium bathing their bodies. (2) The processes by which changes in the external

1. Bottazzi, *Ergebnisse der Physiologie*, Ab. I and II, Jahr. 7, 1908.

media affect the blood. (3) The effects of such changes in the external medium upon the life of the animal. This report deals chiefly with the first of these, and the results tend to show that the blood and coelomic fluids of both aquatic invertebrates and vertebrates are in equilibrium with the external media though perhaps differing very much from it, and any change in the constitution of the latter brings about a gradual change in the blood, to a greater or less degree in the different groups, until a new equilibrium is set up.

Variations in Weight, and Constitution of the Blood of Invertebrates, Accompanying Changes in the External Medium

It is now well known that the blood and internal media of marine invertebrates is practically identical with the sea water in which they are living. Fredericq in 1885[1] showed that by altering artificially the concentration of the sea water, the blood of the animals changed similarly, and stated, moreover, that the time taken for the blood to become once more like the altered external medium, was not the same for different species. Garrey[2] found also that the exposure of Limulus to the drying influence of the atmosphere caused the freezing point to change, and similarly a sojourn in a damp cellar affected the concentration of the blood. He showed further that when other marine invertebrates, Nereis, Homarus, Chaetopterus, etc., were kept in diluted sea water, the concentration of the blood became less, and entrance of water took place, since the animals were frequently swollen after such immersion.

The following experiments indicate the changes in weight that I found to take place with some common marine invertebrates. The animals were all taken direct from sea water, the surplus water removed as far as possible, and then weighed. A control experiment showed that the amount of error due to adherent water was not great enough to influence the results.

1. Fredericq, *Archiv. de Zool. exper.*, 2 série, T. III, 1885.
2. Garrey, *Biol. Bull.*, Vol. VIII, 1905.

Species	Weight when taken from sea water	Time of immersion	Weight after immersion
Cancer pagurus	311 grms.	4 hrs.	320 grms.
(edible crab)	355 grms.	6 hrs.	362 grms.
,,	170 grms.	6 hrs.	180 grms.
,,	302 grms. (two specimens)	3 hrs.	312 grms.
	These two specimens after the three hours in fresh water were replaced in sea water, and after	3 hrs. 20 mins.	305 grms.
Hyas araneus	118 grms. (two specimens)	3 hrs.	123 grms.
(sand crab)	36·5 grms. (two specimens)	1 hr.	38·25 grms.
	These two specimens were now replaced in sea water, and after	2 hrs.	34 grms.
Doris tuberculata	52 grms. (three specimens)	2 hrs.	55 grms.
Arenicola marina	34 grms. (two specimens)	½ hr.	38·5 grms.
(lug worm)	These two specimens were now replaced in sea water, and after	½ hr.	35 grms.

These experiments indicate the rate of increase in weight, due to absorption of water, when marine invertebrates are placed in fresh water; and it will be seen from the above results with the edible crab, the sand crab, and the lug worm, that if they are replaced in sea water after a short interval, before they are seriously injured by the fresh water, they recover, lose water and diminish in weight accordingly.

Variations in Freezing Point.—The following experiments show the effects of a sojourn in hypertonic and hypotonic sea water, on the freezing point of blood and coelomic fluid of invertebrates. The hypertonic sea water was made by adding NaCl, KCl and $CaCO_3$ (in the proportions in which they occur in sea water), to that medium ; the hypotonic by dilution of ordinary sea water with fresh.

Species	Depression of freezing point of blood or coelomic fluid in sea water: (Δ – 1·91°)	Hyper and hypotonic solutions used Δ	Cl per cent.	Time of Immersion hours	Depression of freezing point of blood or coelomic fluid at end of experiment	Cl contents of blood at end of experiment per cent.
Cancer pagurus (blood)	– 1·845° – 1·915°	– 0·575	0·575	11	– 1·402	1·05
Cancer pagurus (blood)	– 1·845° – 1·915°	– 3·070	3·075	11	– 2·785	2·3
Echinus esculentus (sea urchin)	– 1·860	– 0·76	—	3½	– 1·77	*Note.*—Cl per cent. in the normal blood is 1·7 per cent.
(coelomic fluid)	– 1·860	– 2·98	—	3½	– 2·065	
Asterias rubens (starfish)	– 1·860	– 0·965	—	3½	– 1·36	
(coelomic fluid)		– 3·00	—	3½	So much water was withdrawn by the hypertonic solution, that the amount of coelomic fluid was insufficient for determination	

In all cases, in the above experiments the normal equilibrium of the blood or other internal fluid with the sea water was disturbed, and an attempt was made to set up a new one. Eleven hours were insufficient for the blood of the crab to attain its normal relations to the external medium, and any longer immersion than this would have been fatal; just as longer immersion than three and half to four hours would have caused death of the Echinus or Asterias, and also without an equilibrium having been reached.

The osmotic pressure of the blood of invertebrates has been regarded as wholly dependent upon the external medium, that is, identical with it, the relations of the external and internal media being determined simply by osmosis through the body membranes. It is more likely that here also, there is a definite equilibrium between the blood and internal media on the one hand and the sea water on the other, even though normally the osmotic pressure of both is almost identical. Small differences very often occur, and I do not consider these due to errors in observation. Furthermore, it has been often forgotten that the blood of the freshwater crayfish has a Δ of — 0·8° though living in water whose depression of the freezing point may be almost nil. This is a perfect case of an equilibrium between

the blood of an aquatic invertebrate and the external medium, yet both fluids differ considerably in concentration. The freshwater crustacea are, therefore, very similar to the freshwater fishes (Teleostei).

Osmotic Pressure of Blood of Teleosts and Elasmobranchs and Changes Produced by Alterations in the Concentration of the External Media

It has already been shown by me[1] that the concentration of the blood of teleosts is not altogether independent of the external medium.

Thus the teleosts in the fresher water of the Baltic have a slightly lower osmotic pressure for the blood, than the same species in the North Sea. Freshwater teleosts also have a slightly lower osmotic pressure and salt contents than marine species, and the eel (*Anguilla vulgaris*) when placed in sea water was shown to change so that after several hours the osmotic pressure of the blood had risen to about the average for marine teleosts.

The following are the results of experiments made to determine the effects of dilution of the sea water upon the blood.

Pleuronectes flesus (flounder).—This fish though a typical marine flat fish, often penetrates a considerable distance into river estuaries, so that it may even be found in fresh water. It returns, however, to the salt water of the sea at spawning times, and hence resembles the eel to a certain extent, since both move back into the sea—a reversal of the rule of the salmon, which ascends the rivers for spawning.

The freezing point and Cl contents of the blood have been determined for fish caught round Helgoland in water whose $\Delta = -1{\cdot}91^\circ$, and also for some fish caught in the fresh water of the Elbe and forwarded by steamer.

Helgoland.—*P. flesus*—

Δ for blood from caudal artery $-0{\cdot}883^\circ$, $-0{\cdot}903^\circ$, $-0{\cdot}903^\circ$.

River Elbe, Hamburg. *P. flesus*—

Δ for blood from caudal artery $-0{\cdot}68^\circ$.

1. *Bio-Chem. Journ.*, Vol. III, p. 258, 1908.

There is, therefore, a very considerable difference in the osmotic concentration of the blood of the flounder, according as it is living in fresh water or sea water respectively.

The following experiments were all carried out in two large concrete tanks into which sea water or rain water from a reservoir, could be pumped as desired. The rain water was examined and found to be much purer than the fresh water from springs on Helgoland. When fresh water or a mixture of this and sea water was used, the water was well aerated by compressed air driven through pieces of cane. With sea water, the water was also kept running.

Cyclopterus lumpus (the Lump-sucker)—

Normal fish from aquarium tank. Blood $\Delta - 0{\cdot}648^\circ$

Normal fish from aquarium tank. Blood $\Delta - 0{\cdot}658^\circ$

Experiment I.—One specimen placed in a mixture two-thirds fresh and one-third sea water, six hours, then in three-quarters fresh and one quarter sea water for two hours. Killed after eight hours in diluted sea water. Blood $\Delta - 0{\cdot}620^\circ$.

Experiment II.—One specimen placed in diluted sea water ($\Delta - 0{\cdot}70^\circ$) for two hours and finally in fresh water for 24 hours. Blood $\Delta - 0{\cdot}597^\circ$.

Experiment III.—One specimen in diluted sea water ($\Delta - 0{\cdot}37^\circ$) for 48 hours. Blood $\Delta - 0{\cdot}610^\circ$.

In each case a reduction of the osmotic pressure took place, or in other words, a new equilibrium was either set up or in process of being set up. Taking the mean for the normal blood as $\Delta - 0{\cdot}653^\circ$, the amount of change in the three experiments was $-0{\cdot}033^\circ$, $-0{\cdot}056^\circ$, and $-0{\cdot}043^\circ$ respectively.

The coelomic fluid and fluid from the stomach of the last specimen of Cyclopterus was also examined in order to determine their relations to one another and to the external medium. The results were :—

	From a normal fish in sea water—
Fluid from coelom $\Delta - 0{\cdot}617^\circ$	Fluid from coelom $\Delta - 0{\cdot}690^\circ$
Fluid from stomach $\Delta - 0{\cdot}36^\circ$	Fluid from stomach $\Delta - 1{\cdot}593^\circ$

Thus, the coelomic fluid has its own equilibrium, the freezing point being a little lower than that of the blood but very similar. The contents of the stomach on the other hand have a Δ which is directly dependent upon the external medium swallowed, the

food and secretion. The very different Δ's of the coelomic fluid and contents of the alimentary canal in the last experiment are striking, since both are only separated by the thin walls of the intestine.[1]

The Eel. Anguilla vulgaris.—In my previous paper, the results of some experiments made on the eel, taken from fresh water, were given, and it was shown that the concentration of the blood increased when the fishes were placed in sea water.

The blood of three freshwater species then examined gave the following figures :—

Carp	$\Delta - 0{\cdot}487^\circ$
Abramis brama	$\Delta - 0{\cdot}510^\circ$
Eel	$\Delta - 0{\cdot}570^\circ$

After twenty-four hours in concentrated sea water, sp. gr. 29·3, the Δ of the blood of the eel had increased to $-0{\cdot}745^\circ$.

The following supplementary experiments have been carried out with the same species of eel, but caught in the sea off Helgoland :—

Blood from normal specimens taken direct from sea water ...	$\Delta - 0{\cdot}649^\circ$
" " " " ...	$\Delta - 0{\cdot}620^\circ$

These figures are very interesting, for in the first place they confirm the aquarium experiment at Kiel in showing that the concentration and osmotic pressure of the blood of the eel is greater in the sea than in fresh water, and further show that the osmotic pressure reached by the blood ($\Delta - 0{\cdot}745^\circ$) in the Kiel experiment was too high. Perhaps the rapid change from fresh water to sea water at Kiel caused the blood to pass beyond the normal equilibrium

1. It had been noticed that when marine fish were kept in fresh water, there was a gradual, but small, lowering of the osmotic pressure until death. Just before death, however, the diminution was considerable. It was in fact as if the teleost resisted the action of the external medium as far as possible. This was partially confirmed by a determination of the freezing point of a *Cyclopterus* in aquarium water, but in a pathological condition.

Cyclopterus appears in the shallow water round Helgoland, often adhering to the rocks and left dry by the tide, during the spawning season in spring only. In May and June it begins to disappear, and in summer seems to have left for deep water, since very few are caught. The fish kept in the aquarium, though living well in spring, almost all die as the time approaches when they should normally leave for deep water. Death comes gradually, and the jaws and bony scutes appear to decay, so that the fish presents for some time a very unpleasant appearance.

The freezing point of the blood of all the normal specimens of Cyclopterus was about $-0{\cdot}658^\circ$. One of these pathological specimens which was examined had the remarkable value $\Delta - 0{\cdot}765$, or $-0{\cdot}107^\circ$ more than the normal. It may be said, therefore, that the concentration of the blood of pathological specimens tends to approach that of the fluid in which they are living.

point for sea water, and after a longer immersion in that water it would probably have attained the more normal $\Delta - 0{\cdot}634^\circ$, the mean for those specimens taken direct from the sea at Helgoland.

As converse experiments to those at Kiel, some specimens were placed in aerated fresh water, the change being gradual.

Eels placed direct in mixture half sea and half fresh water, and fresh water pumped in at intervals until the whole was perfectly fresh. The eels finally remaining in fresh water for three days.

Results :—(*a*) Blood $\Delta - 0{\cdot}580^\circ$
(*b*) Blood $\Delta - 0{\cdot}583^\circ$
(*c*) Blood $\Delta - 0{\cdot}582^\circ$

Thus the osmotic pressure had decreased, and the freezing point ascended until it was practically the same as that recorded at Kiel for the fish from fresh water.[1]

All the experiments, therefore, made on the teleosts show that the blood is in equilibrium with the sea water or the fresh water in which the fish are living, and a change in the external medium is followed by processes setting up a new equilibrium. This change may or may not prove fatal to the animal. The relation of the osmotic pressure of the blood of teleosts to the external medium is very like the relation of the temperature of mammals to their surroundings. In the mammals the temperature is normally nearly constant, yet not altogether independent of the temperature of their environment, for a great rise or fall in the temperature of the latter produces a corresponding but smaller change in that of the body. In short, the temperature of the body is in equilibrium with the temperature of the surroundings though both may or may not be identical.

1. It is interesting to note that the freezing point for the eels from the sea is the *highest* of any marine teleostean examined by me, and further that the freezing point of the blood taken from freshwater specimens was *lower* than that from the other species experimented with. Hence, under normal conditions, the concentration of the blood fluctuates but little, compared with the change it would have to pass through if the blood of eels from sea water had an osmotic pressure equal to the average for marine teleostei. This feature can be correlated perhaps with the power the eel has of resisting sudden changes in the constitution of the external medium.

Elasmobranchs

The blood of these fishes has usually the same osmotic pressure as the surrounding sea water. Experiments were made at Helgoland to determine how long elasmobranchs could live when transferred directly to fresh water, and the condition of the blood just before death. Mosso,[1] states that sharks die after some hours, and further, that after half an hour in fresh water, no more blood flows out from the caudal artery if the tail be cut, even though the heart pulsates. He states further that if a solution of NaCl is injected it will not pass through the gills, showing they are no longer permeable and that the blood of the fishes dead in fresh water is almost normal, death being due to stoppage of the gill capillaries. The first experiments were performed with very large *Acanthias vulgaris* (piked dog fish).

Average Δ for blood of normal fish from sea water $-$ 1·90° and Cl 0·88 per cent.

I.—Specimen placed directly into fresh water and after 4 hours 10 minutes blood taken.

The fish was nearly dead, but even after this long period blood was easily obtained from the tail and gave Δ $-$ 1·455° and Cl 0·48 per cent.

II.—*Acanthias vulgaris.* Placed directly in fresh water—
Blood abstracted after 3 hours 45 mins. Δ $-$ 1·448°.

III.—*Acanthias vulgaris.* Placed directly in fresh water—
Blood abstracted after 4½ hours Δ $-$ 1·400°, Cl 0·455 per cent.

In all these cases, after more than three hours in fresh water, blood was easily obtained from the tail, though not in such large amounts as from the normal fish. Moreover, it can hardly be said from the above results, that at the time of death almost no change had taken place in the blood. There is no doubt, however, that the result varies greatly with the species, and perhaps that experimented with by Mosso was more like the Rays, the results of which follow.

With regard to the power of resisting fresh water, Acanthias almost if not quite equals the cod in this respect, and if the cod only was used as typical of the teleosts, and Acanthias as an elasmobranch, the great difference existing between these groups would not be so conspicuous.

1. Mosso, *Biol. Centralblatt*, 1890.

Another interesting point in the above results is that the reduction in the salt contents of the blood, as indicated by the chlorine contents, is much greater than the lowering of the osmotic pressure would lead one to expect.

Experiments with other Elasmobranchs.—

Raia clavata.—Average Δ for blood from normal specimens, = $-$ 1·90°.

One large specimen was placed in well aerated fresh water. This fish secreted large quantities of mucus, and at the end of two hours was almost dead. The heart was still beating, and blood was obtainable from the tail, but the quantity was small.

Δ of blood $-$ 1·645°

An elevation of the freezing point had thus taken place, though at the time of death it was much lower than that of the Acanthias blood.

The same speedy death was exhibited by other specimens of the same species. Two specimens (small) of *Raia batis* died within two hours after direct immersion in fresh water, but not enough blood was obtained from the caudal artery to allow of a determination of the freezing point.

At the suggestion of Dr. Franz, some of the fluid was taken from the eyes of Acanthias. These were removed carefully from the fish, the sea water removed, and the fluid abstracted. The freezing point was remarkably low, lower even than that of the blood, viz. Δ — 2·028°, and the amount of chlorine, 0·946 per cent., was greater.

The flesh of elasmobranchs, like that of teleosts, contains much less salt than the blood, the figure for the muscles being only 0·15 per cent.

Some very interesting experiments made by Baglioni[1] show that to sustain the normal beat of the heart from an elasmobranch (*Scyllium catulus*), an artificial solution containing 2 grammes urea and 2 grammes NaCl for every 100 c.c. of tap water (in which calcium is present) is necessary. The urea aided contraction and the

1. Baglioni, *Zeit. für allgem. Physiol.*, 1907.

NaCl regulated the relaxation. Hence the urea is present not only to bring the osmotic pressure up to that of sea water, but as a necessary constituent of the blood, without which the regular beating of the heart is impossible. It follows, therefore, that the alterations in the constitution of the blood in the preceding experiments would interfere with physiological processes in addition to any blocking of the gill capillaries that occur as stated by Mosso.

The average results of the resistance of elasmobranchs and teleosts to fresh water, show that the latter can resist much greater changes than the former, and this agrees with the distribution of the elasmobranchs for they are typically marine fish and do not extend far into the Baltic. Bridge[1] states that some species are permanent inhabitants of fresh water; it would be most interesting to determine the freezing point and salt contents of the blood of these representatives of a group so characteristic.

Variations in the Blood of *Pleuronectes platessa* (Plaice) when Transferred from the Sea to the Aquarium

Some experiments were made to observe the change in the osmotic pressure of the blood of this marine teleost when immersed in diluted sea water the osmotic pressure of which was not less than that of the blood of the normal fish. These experiments led to a remarkable change being discovered, which took place under apparently normal conditions in the aquarium. The first experiments were as follows :—

A number of large plaice, caught off Helgoland, were brought back living, and eight set in a mixture of sea and fresh water, the proportions being about two-thirds sea and one-third fresh. After two hours this was changed by addition of more fresh water until the Δ was $-1{\cdot}125°$, and the fish remained in this for fourteen hours.

Blood from three plaice, out of this mixture $\Delta -0{\cdot}640°$
Blood from eight plaice, out of this mixture $\Delta -0{\cdot}630°$

Blood from plaice out of same catch but kept in normal sea water in aquarium :—

Blood from three specimens $\Delta -0{\cdot}665°$
Blood from four specimens $\Delta -0{\cdot}695°$

Two things are noteworthy here : (1) The osmotic pressure

1. Bridge, *Cambridge Natural History*, Vol. VII, London, p. 432, 1904.

of the blood has decreased in this diluted sea water ; (2) the osmotic pressure of the blood of those fishes kept in the aquarium, but in sea water, is much lower than the figures obtained previously[1] for the same species direct from the sea.

A second experiment was consequently performed ; a considerable number of fishes were brought back, and divided into two lots.

First Lot.—Examined immediately. Blood gave—
For three specimens $\Delta - 0{\cdot}715^\circ$
For three specimens $\Delta - 0{\cdot}710^\circ$
For four specimens $\Delta - 0{\cdot}705^\circ$
Second Lot.—Kept in *sea water* in aquarium for 34 hours.
Average result blood $\Delta - 0{\cdot}630^\circ$

That is, for those specimens in sea water, under apparently normal conditions in the aquarium, the freezing point was higher by $-0{\cdot}08^\circ$ than the specimens examined immediately after capture.

A third experiment was made to confirm the two previous ones by taking some of the blood from the fishes just after capture, the salinity of the bottom water being determined at the same time. The catch was divided into three lots. Two of these were brought back alive to land, the blood was taken from the others and brought back in tubes kept in ice. Of the two lots brought to land, one was examined immediately, and the other lot placed in an aquarium tank. The salinity of the bottom water and the aquarium tank water was about the same, viz. : Bottom water 36·55 per thousand, Aquarium water 36·00.

Results :— Blood taken from fishes immediately trawl was brought on deck—
First Series— $\Delta - 0{\cdot}735^\circ$
Second Series— $\Delta - 0{\cdot}753^\circ$
Third Series— $\Delta - 0{\cdot}700^\circ$

Blood taken from fishes of same catch brought back to the Biological Station in running sea water. Time taken on way, $2\frac{1}{2}$ hours—
First Series— $\Delta - 0{\cdot}741^\circ$
Second Series— $\Delta - 0{\cdot}742^\circ$

Blood taken from fishes of same catch brought back to the Biological Station in running sea water, and kept for three days in large aquarium tanks—
First Series— $\Delta - 0{\cdot}645^\circ$
Second Series— $\Delta - 0{\cdot}655^\circ$

1. Dakin, *Bio-Chem. Journ.*, Vol. III, p. 264, 1908.

It will be seen from the above series of experiments that on every occasion there was a very considerable alteration in the freezing point after the fishes had been kept in the aquarium. Moreover, there was but little variation in these results; the fishes taken from the sea water direct had almost all the same Δ for the blood, those kept in the aquarium also agreed between themselves, but differed, as the results show, very much from those examined directly they were brought to land.

This remarkable change could not have been due to differences in salinity of the water, since analyses showed the salt contents was practically the same. If the fish had been pathological or weak through the change, the freezing point for the blood would be expected to have been lower, that is, more like the sea water. Instead of this, it was much higher in the aquarium fish, and they were quite healthy.

It is possible that this change was due to the difference in hydrostatic pressure, since in the sea the fish must have been living under about four atmospheres pressure. It is obvious that this would have no effect directly, but it may be responsible indirectly for changes resulting in the blood.

It was not possible at Helgoland to determine whether this change took place generally with fishes living on the sea bottom, when brought into the aquarium, but I hope to continue this series of experiments shortly.

I have, in order to compare the action of fresh water on marine teleosts, in this paper compared the results with the freezing points of blood from fish in the sea water tanks of the aquarium, though it does not seem from the figures for Cyclopterus that they differ from those caught in the sea. In any case, it will be seen that great care must be taken when examining the blood for purposes of comparison, to make certain the fishes are under the same conditions in each case.

Influence of the Osmotic Concentration of the Sea Water or the Blood upon Pelagic Eggs

The eggs of many marine teleosts are pelagic, and their specific gravity is so adjusted that but slight changes in the density of the water, if the salt contents is reduced, are sufficient to cause the eggs to sink.

Thus the eggs of the plaice or other pelagic eggs found floating twenty miles west of Helgoland, sink in the slightly less dense water round that island. In the Baltic Sea, where the difference between the *surface* and *deep* water in salt contents is very considerable, the eggs are very rarely found floating at the surface, but remain suspended at some point nearer the bottom. It has been found from measurements made chiefly by Ehrenbaum and Strodtmann,[1] that the diameter of these pelagic eggs is greater, the less dense be the sea water (with one exception—the plaice).

For example, the eggs of the flounder from the Baltic Sea had a diameter of 1·054 and 1·216 mm., and they occurred in water with salt contents 11·24 to 17·54 per thousand. The eggs of the same species from the North Sea had a diameter of 0·915 and 0·970 mm. It is still more interesting to find that the eggs while still in the ovary are larger in Baltic Sea specimens than in those taken from the salter North Sea water. For example, the ovarial eggs of *Clupea sprattus* have, according to Schneider, a diameter of 1·2 mm. in Baltic Sea specimens. The diameter of the eggs of the same species in the North Sea is, however, much less than this, and since these extruded eggs are larger than the ovarial, the ovarial eggs must be still smaller than those from fishes in the Baltic waters.

Ehrenbaum gives 0·94 mm. as the diameter of the ovarial eggs of *Pleuronectes limanda* in the Baltic Sea, whilst the floating eggs of this species in the North Sea only reached a diameter of 0·840.

The probability of this difference in size being correlated with the difference in salt contents of the blood was first suggested by Ehrenbaum and Strodtmann, who said: 'Vielleicht beeinflusst das

1. *Wiss. Meeresuntersuch*, Abt. Helgoland, Bd. VI, H. 1, 1904.

umgebende Wasser die Fische in der Weise, dass in der Blutflüssigkeit ~~und vor~~ allen Dingen in dem Liquor sich der Salzgehalt ändert. In der östlichen Ostsee befinden sich die Fische in der Regel in einem Wasser von geringem Salzgehalt, und hierdurch erhält auch die die Eier umgebende Flüssigkeit des Ovars eine geringe Konzentration. Ist nun die zur Bildung eines Eies erforderliche Salzmenge eine konstante, so wird in diesem Falle die enstehende osmotische Spannungsdifferenz eine Vergrösserung des sich bildenden Eies bewirken. Die Volumenzunahme hat dann den Vorteil, das spez. Gewicht zu verringern, so dass die Eier auch noch in weniger salzhaltigem Wasser zu schwimmen vermögen.'

This suggestion has been justified, since I found that the salt contents of the blood does differ, though slightly, in the fishes from sea water of varying density. The ovarial fluid, like the blood, has a salt contents much lower than that of the surrounding water. It is usually slightly lower even than the blood, and also dependent to a small extent, like that fluid, on the concentration of the sea water. These differences might account for differences in the size of the eggs in the ovary, but they would not account—they are so small—for differences in the specific gravity of the eggs from two places so near, as previously mentioned. Ehrenbaum and Strodtmann state, moreover, that by taking fertilized eggs of Ctenolabrus and Sprat, from North Sea water, gradually down to almost fresh water, no noticeable increase in size was found to take place. Hence, I think, the specific gravity of the eggs is determined to a large extent by the density of the water in which the fish are living at the moment of extrusion of the eggs, when an increase in size takes place; and the larger size of the eggs in the ovary in fishes living in much less dense water is due to the action of the outer medium on the blood and ovarial fluid.

Conclusion

The osmotic pressure of the blood of teleosts is, as we have seen, subject, though to a small extent only, to changes in the density

of the outer medium.[1] The river eel has a lower osmotic pressure for the blood in fresh water than in sea water, though the Δ for this fish from sea water is higher than that of any other marine teleost. Moreover, there is a greater percentage of salt in the blood of marine teleosts, and Sumner considers that both water and salts may pass osmotically through the bounding membranes.

He states[2] that control experiments excluded the possibility that the water or salts passed from the body through the alimentary canal, leaving as the only possible alternative an osmotic exchange through one or more of the limiting membranes. Further, he remarks (p. 97) 'The lining of the alimentary canal is, of course, readily permeable to fluids and to various substances in solution, but this, it is needless to say, is not freely exposed to the surrounding medium.' Now I do not consider the alimentary canal excluded, since water must pass in with the food; and my freezing point determinations on the fluid of the alimentary canal of Cyclopterus show that in sea water this fluid has a salt contents almost the same as sea water. Again, when Cyclopterus was immersed in fresh water, the fluid in the alimentary canal had a Δ much less than the blood and rather approaching the surrounding water in composition. This being the case, there is present the possibility of water or salts passing though the membranes of the alimentary canal by simple osmosis or by the more complicated chemical processes of physiological absorption.

On the other hand Sumner showed that keeping freshwater

1. The low osmotic pressure of the teleost blood, and its small range of variation compared with that of invertebrates, has led Dekhuyzen to suggest (*Ergebnisse von osmotischen Studien*, *Bergens Museums Aarbog*, 1904, No. 8, p. 7), the probability of the teleostean fishes having descended from Ganoids which lived in brackish water. They are assumed to have taken the osmotic pressure of the water there, and then having evolved the power of keeping this constant, wandered on the one hand to the sea and on the other to fresh water.

One would then have to assume also that the elasmobranchs had inherited the salt contents of their blood from ancestors living in brackish water, because though the osmotic pressure is the same as the surrounding medium, the salt contents is only about half that of the North Sea water.

It is much more likely that the teleostei have acquired the low osmotic pressure in the sea. Perhaps, as suggested by some authors, it represents the concentration of the primeval seas, which had a much lower density; or what is more probable, since all the vertebrates from teleosts to mammals have evolved the power of maintaining a definite salt contents for their blood, in defiance of solutions possessing higher or lower concentrations bathing their bodies, and passing through their alimentary canals, it is possible that at the same time the teleostean fishes acquired this low salinity, which is characteristic of vertebrates even though living in water of much higher concentration.

2. *Bull. of the Bureau of Fisheries*, p. 104.

fish with the body immersed in fresh water, but passing salt water over the gills, caused death, and there was a considerable loss of weight. No experiments, however, that have been made prove the permeability of the membranes, e.g. gills, for both water and salts by simple osmosis, though it seems certain that they are permeable for water, which would suffice to a certain extent for the toxic action of the salt water on the gills of freshwater fish. Botazzi and Enriques,[1] experiments indicate an impermeability of the alimentary canal walls of invertebrates to salts in solution except by nutritive processes, and Overton[2] after a number of experiments on the frog, as an example of the Amphibia, concludes that the skin is only with difficulty penetrated by salts, though very permeable for water. Schücking,[3] on the contrary, finds that salts leave the body of Aplysia, when it is immersed in fresh water, though the mouth and anus were ligatured, and that the amount of water or salts lost or gained is affected by injection of small quantities of nicotine, strychnine, etc. He concludes that the membranes are permeable to both water and salts. It is impossible to say whether the increase in salt contents of the blood of teleosts, in water of greater concentration, is due to the passage of salts through the gills by diffusion or through the walls of the alimentary canal by diffusion or absorption. The sensitiveness of the teleosts to changes in the chemical constitution of the external medium, is indeed apparent, and it appears that the toxicity of fresh water for marine teleosts, and vice versa, is due more to the alteration in the chemical constitution than to differences of osmotic pressure, though in all probability both are concerned.

This certainly points to a permeability for salts, but does not indicate whether through the gills by diffusion, or the alimentary canal by absorption. Under normal conditions the Δ of the urine of the teleosts is *less* than that of the blood, yet at the same time the kidneys appear to regulate to a certain extent the concentration of the blood, for it was generally found that marine fishes immersed in fresh water excreted large quantities of urine.

1. *Archiv. f. Anat. u. Physiologie, Abt. Physiol.*, Supplement-Band., 1901.
2. Overton, *Verhandlung. der Physik-Medic. Ges. zu Würzburg*. N.F., Bd. XXXVI, No. 5, 1904.
3. Schücking, *Archiv. f. Anat. u. Physiol.*, 1902.

Whatever be the path taken by the water and salts, it is evident that a passage of both does take place both in vertebrates and invertebrates, and further that under normal conditions in fresh or sea water the blood and other fluids of the body are in equilibrium with the external medium. This condition is very like that suggested by Moore and Roaf[1] for the qualitative differences between the electrolytes of the cell and those of its environment, an equilibrium consisting not necessarily in an equality or isotonism of the total osmotic pressure.

It has already been pointed out that the relation of the osmotic pressure of the blood of teleostean fishes to the water in which they live, is very like the relation of the temperature of 'warm blooded' vertebrates to that of their environment. This comparison might be carried still further and the aquatic invertebrates likened to the so-called 'cold blooded' animals whose temperature is practically identical with that of their environment.

Finally, it has been shown that any change in the chemical constitution of the surrounding water brings about for both invertebrates and vertebrates, sooner or later, an alteration in the electrolytes and non-electrolytes of the internal fluids. This is followed by an alteration of the chemical constitution of the cell, which draws inseparably after it an alteration or disturbance of its function.

I should like here to express my thanks to Professor Heincke, Director of the Biological Station on Helgoland, for his kindness in allotting me a work place and for providing me with the large quantity of material necessary, and also to the Staff of the Station, and in particular Professor Ehrenbaum for his interest and helpful suggestions. I have also to thank Professor Benjamin Moore for his many suggestions.

1. Moore and Roaf, *Bio-Chem. Journ.*, Vol. III, p. 55, 1908.